Short-Chain Peptide-Dye Conjugates as Contrast Media for Optical Diagnosis

The invention relates to compounds for tumor diagnosis that consist of conjugates of dyes with short-chain peptides, which are derived from vaso-active intestinal peptide, somatostatin or neurotensin, the use of these compounds as optical diagnostic agents, and diagnostic agents that contain these compounds.

On a cellular level, disease-induced alterations are often manifested as a receptor distribution or expression that is altered relative to the normal state. These differences can be both of quantitative type (e.g., the amount of transferrin receptors in proliferating cells) or else also of qualitative type (e.g., expression of vascular endothelial growth factors, VEGF). Previous attempts to image a pathological receptor expression or distribution have been noted mainly in radiodiagnosis because of the necessary sensitivity of the detection process.

Heptahelical receptors are target molecules of many pharmacological active ingredients (e.g., β-blockers, H2-acid blockers, antihistamines). In addition to therapeutic batches, mainly radiolabeled, agonistic ligands of these receptors are

used diagnostically for the so-called receptor scintigraphy for in-vivo detection and location of tumors. In this case, the mechanism of the receptor-mediated endocytosis is used, e.g., by the somatostatin receptor, which is more strongly expressed in neuroendocrine tumors. The somatostatin analog ^{111}In-DTPA-pentetreotide (octreoscan$^{(R)}$) is clinically approved for routine scintigraphic diagnosis; literature: J. Steroid Biochem. Mol. Biol. 37, 1079-82, 1990, J. Nucl. Med. 32, 1184-9, 1991,; J. Nucl. Med. 33, 652-8, 1992; Digestion 3, 54-9, 1994, J. Clin. Invest. 93, 1321-5, 1994, Metabolism 45, 21-3, 1996.

Another batch consists in the use of radiolabeled VIP and VIP-analogs, which bind to the VIP-receptors. The VIP-receptor is more strongly expressed by a broad spectrum of tumors (i.a., adenocarcinomas).

WO 96/30055 describes radiodiagnostic and radiotherapeutic reagents, special VIP-receptor-binding peptides, which are radiolabeled and can be used for radiodiagnosis and radiotherapy. VIP-receptor-binding peptides that can be labeled with Tc-99m for scintigraphy are described especially advantageously. Additional literature: Cancer Research 54, 690-700, 1994; Endocrinology 136, 2662-80, 1994, J. Nucl. Med. 40, 353-361, 1999.

All described diagnosis batches that are based on the somatostatin receptor and VIP-receptor are radiodiagnostic

batches (scintigraphy with ^{123}I, ^{125}I, ^{111}In or ^{99m}Tc-labeled peptides).

Literature: EP 588754, US 5650134; US 5620675; US 5225180; WO 96/23527; J. Steroid Biochem. Mol. Biol. 37, 1083-87, 1990; Lancet 242-4, 1989, J. Nucl. Med. 39, 1913-17, 1998.

No fluorescence-labeled peptides that are conjugated with dyes that make possible an in-vivo-fluorescence detection of tumors are known to date, however (Photochem. Photobiol. 68, 603-632, 1998).

The object of the invention is to make available new compounds that make possible a sensitive diagnosis of tumors by detection of fluorescence radiation with use of a receptor-specific binding of the compounds to the target tissue. In this case, special dye molecules that are coupled to biomolecules are to yield a highly sensitive, detectable fluorescence signal.

The object is achieved by the provision of compounds that contain fluorescence dyes, which are coupled covalently to short-chain peptides. These conjugates have a high binding affinity to heptahelical receptors, especially the somatostatin receptor, the VIP-receptor (vaso-active intestinal peptide), and the neurotensin receptor, and they are optionally taken up intracellularly by receptor-mediated endocytosis. The compounds according to the invention are therefore suitable for the technically simple, harmless optical diagnosis of tumor cells and tumor tissues, which increasingly express somatostatin receptors,

VIP-receptors or neurotensin receptors in comparison to healthy cells. Especially suitable are the compounds for fluorescence diagnosis and especially advantageously for the fluorescence-endoscopic diagnosis in hollow organs, such as the esophagus, the cervix, the colon, and the bronchial tubes of various tumor types, such as, e.g., adenocarcinomas, neuroendocrine tumors or ductal pancreatic tumors.

Especially preferred dyes are distinguished in that they satisfy certain photophysical and chemical requirements. From the photophysical standpoint, the dyes must have high absorption coefficients and high fluorescence quantum yields to provide an effective signal even in the case of the smallest tissue concentrations. The absorption maxima must overlap a wide spectral range in a freely selectable manner. Thus, for detection in lower tissue layers (several centimeters below the surface), the spectral range of between 600 and 900 nm is essential, while for surface detection, absorption wavelengths of 400 to 600 nm are sufficient. From the chemical standpoint, the dyes must have a high photostability and must exhibit no signs of decomposition (photobleaching) during excitation. The dyes must be usable as synthesis components in the solid-phase-synthetic production of peptides and thus be stable under common synthesis conditions so that a simple, advantageous production of structurally defined dye-peptide-conjugates with solid stoichiometric ratios is ensured between dye and peptide. The

requirements are best satisfied by polymethine dyes, especially cyanine, merocyanine, oxonol and squarilium dyes.

Subjects of the invention are therefore peptide-polymethine dye-conjugates of general formula (I)

$$A^1 - (X)_m - A^2 \qquad I$$

in which

X stands for an α-, β- or γ-amino acid with D- or L-configuration, and

m stands for a number from 5 to 30, whereby the resulting amino acid sequence $(X)_m$ can be cyclized in a straight-chain nature or via a disulfide bridge between two cysteines or homocysteines or amidically between the N- and C-terminus and stands for the amino acid sequence of the vaso-active intestinal peptide (VIP), the somatostatin or the neurotensin, or for fragments, partial sequences, derivatives or analogs of the VIP, somatostatin or neurotensin,

A^1 stands for a hydrogen atom, an acetyl radical or an alkyl radical with up to 10 C atoms, which optionally can be substituted with 1 to 3 carboxy groups and/or 1 to 6 hydroxy groups, or a poly(oxyethylene) radical with 2 to 30 $-CH_2CH_2O$ units, or a dye molecule from the class of the polymethine dyes, which has at least one absorption maximum in the range of 380 to 1200 nm,

A^2 stands for a hydroxy group, an amino group or a dye molecule from the class of polymethine dyes, which has at least one absorption maximum in the range of 380 to 1200 nm,

under the condition that at least one of radicals A^1 or A^2 represents a dye molecule from the class of polymethine dyes, which has at least one absorption maximum in the range of 380 to 1200 nm,

whereby for the case that A^1 and/or A^2 represents a dye molecule from the class of polymethine dyes, which has at least one absorption maximum in the range of 380 to 1200 nm, A^1 is linked to the N-terminal amino group, and A^2 is linked to an amino group of the amino acid lysine or to a hydroxy group of the amino acid serine in any position within the amino acid sequence $(X)_m$,

and their physiologically compatible salts.

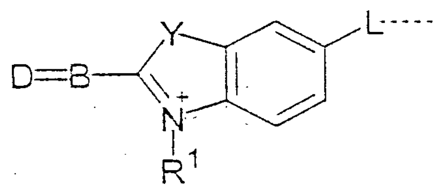
Fragments, partial sequences, derivatives or analogs of the above-mentioned peptides stand, i.a., for shortened amino acid sequences, exchanges of individual or all amino acids for the corresponding D-amino acids, exchanges of individual amino acids for other amino acids, inverted sequences and combinations of the above-mentioned features.

The fragments, partial sequences, derivatives or analogs of the above-mentioned peptides can also contain amino acids that are not natural, such as, e.g., naphthalanine, cyclohexylalanine,

norleucine, norvaline, α-aminoadipic acid, α-aminobutyric acid, β-alanine, β-cyclohexylalanine, ornithine, sarcosine or δ-hydroxylysine.

Especially preferred embodiments of the invention are compounds of general formula I, which are distinguished in that dye molecule A^1 and/or A^2 stands for a cyanine, squarilium, croconium, merocyanine or oxonol dye. These dyes belong to the class of polymethine dyes and have the advantages that are described above.

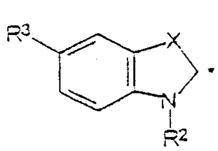
Other preferred compounds of general formula I according to the invention are distinguished in that dye molecule A^1 and/or A^2 stands for a cyanine dye or squarilium dye of general formula II



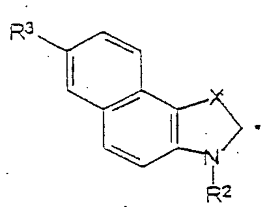
II

in which

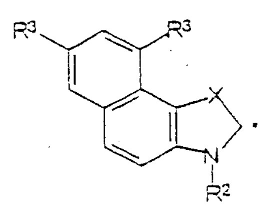
D stands for a fragment corresponding to general formulas III to VI, whereby the position that is identified with a star means the linkage with B



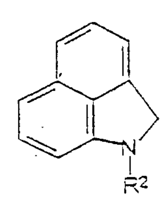
III



IV

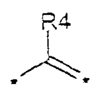


V

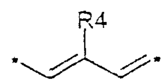


VI

B stands for a fragment that corresponds to general formulas VII to XII



VII



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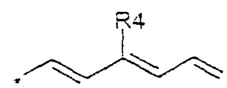
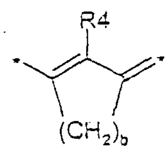
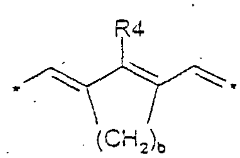


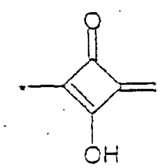
IX



X



XI



XII

R^1 and R^2 stand for E^1, R^3 stands for a fluorine, chlorine, bromine, iodine atom or a nitro group or for a radical $-COOE^1$, $-CONE^1E^2$, $-NHCOE^1$, $-NHCONHE^1$, $-NE^1E^2$, $-OE^1$, $-OSO_3E^1$, $-SO_3E^1$, $-SO_2NHE^1$, $-E^1$,

whereby E^1 and E^2, independently of one another, stand for a hydrogen atom, a C_1-C_4 sulfoalkyl chain, a saturated or unsaturated, branched or straight-chain C_1-C_{50} alkyl chain, whereby the chain or parts of this chain optionally can form one or more aromatic or saturated cyclic C_5-C_6 units or bicyclic C_{10} units, and whereby the C_1-C_{50} alkyl chain is interrupted by 0 to 15

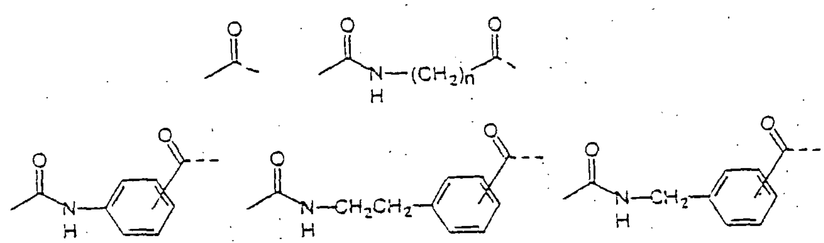
oxygen atoms and/or by 0 to 3 carbonyl groups and/or is substituted with 0 to 5 hydroxy groups,

R^4 stands for a hydrogen atom, for a fluorine, chlorine, bromine, iodine atom or a branched or straight-chain C_1-C_{10} alkyl chain,

b means a number 2 or 3,

X and Y, independently of one another, mean O, S, Se, -CH=CH- or $C(CH_3)_2$,

L stands for a group that corresponds to the formulas below



in which n means a number from 1 to 10.

(In the above-mentioned formulas, the solid line that is drawn to the left in the molecule portion represents the linkage to the dye skeleton, and the dashed line to the right in the molecule portion represents the linkage to the peptide.)

From the class of polymethine dyes, the cyanine dyes, e.g., the indocarbo-, indodicarbo- and indotricarbocyanines that are based on the indole structure, are especially advantageous.

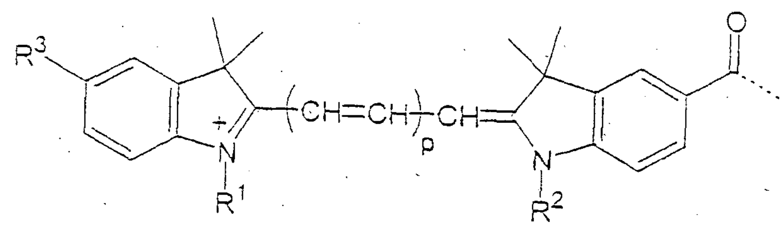
These structures are distinguished by a high chemical and photochemical stability. By advantageous synthesis, derivatives can be obtained that absorb and fluoresce between 400 and 1000 nm in any way desired, can be coupled to peptides by substitution with suitable linkers and functional groups, preferably carboxyl groups, and have a high water solubility, preferably by sulfonate groups. In contrast to cyanine dyes that are known in the literature, the compounds that are used according to the invention have only one reactive group, which makes possible a stoichiometrically defined coupling to the peptide as part of the resin synthesis of the conjugate.

Especially preferred embodiments of the compounds of general formula I according to the invention are therefore distinguished in that

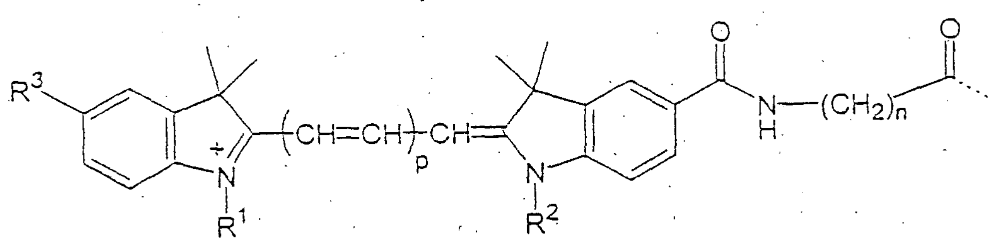
-- dye molecule A^1 and/or A^2 stands for an indocarbocyanine dye, an indodicarbocyanine dye or an indotricarbocyanine dye,

-- dye molecule A^1 and/or A^2 stands for an indocarbocyanine dye, an indodicarbocyanine dye or an

indotricarbocyanine dye of general formula XIII or XIV



XIII



XIV

in which

p stands for 1, 2 or 3,

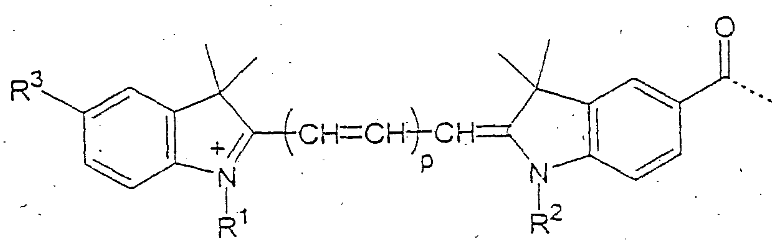
n stands for a number 1, 2, 3, 4 or 10,

R^1 and R^2, independently of one another, stand for a 4-sulfobutyl, 3-sulfopropyl, 2-sulfoethyl, 3-methyl-3-sulfopropyl, methyl, ethyl or propyl radical, and

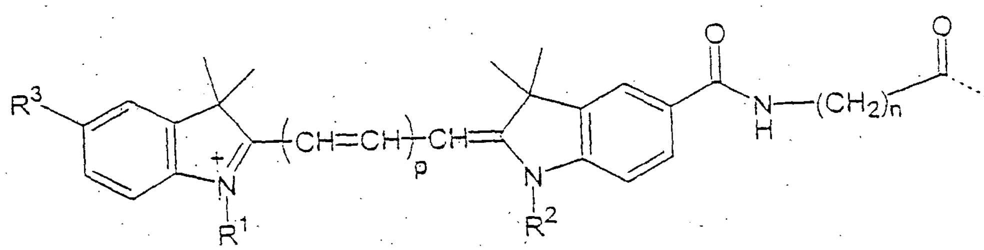
R^3 stands for hydrogen, a chlorine, bromine, iodine atom or a nitro group or for a radical $-COOE^1$, $-CONE^1E^2$, $-NHCOE^1$, $-NHCONHE^1$, $-NE^1E^2$, $-OE^1$, $-OSO_3E^1$, $-SO_3E^1$, $-SO_2NHE^1$,

whereby E^1 and E^2, independently of one another, stand for a hydrogen atom or for a methyl or ethyl radical or a C_3-C_6 alkyl radical, which is interrupted by 0 to 2 oxygen atoms and/or by 0 to 1 carbonyl groups, and/or is substituted with 0 to 5 hydroxy groups, or E^1 and E^2 stand for a poly(oxyethylene)glycol radical with 2 to 30 $-CH_2CH_2O$ units,

-- dye molecule A^1 and/or A^2 stands for an indocarbocyanine dye, an indodicarbocyanine dye or an indotricarbocyanine dye of general formula XIII or XIV



XIII



XIV

in which

p stands for 1, 2 or 3,

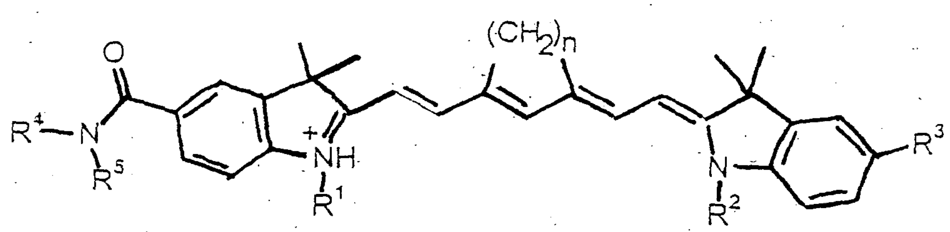
n stands for 1, 2 or 4,

R^1 and R^2, independently of one another, stand for a 4-sulfobutyl or 3-sulfopropyl radical,

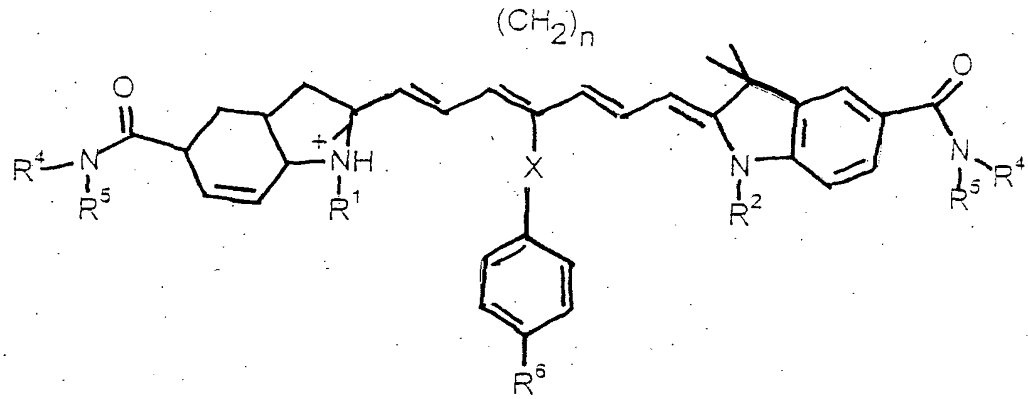
R^3 stands for hydrogen or for a radical $-COOE^1$ or $-CONHE^1$,

whereby E^1 means a hydrogen atom or a methyl or ethyl radical or a C_3-C_6 alkyl radical, which is interrupted by 0 to 2 oxygen atoms and/or by 0 to 1 carbonyl groups and/or is substituted with 0 to 5 hydroxy groups,

-- dye molecule A^1 and/or A^2 stands for an indotricarbocyanine dye of general formula XV or XVI:



XV



XVI

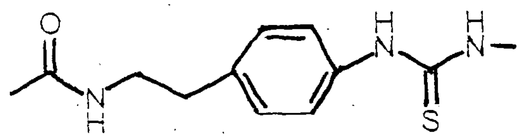
in which

n stands for 2 or 3,

R^1 and R^2, independently of one another, represent a 4-sulfobutyl, 3-sulfopropyl or 2-sulfoethyl radical,

R^3 stands for a radical -CONH-peptide, -CONH-$(CH_2)_m$-CONH peptide, -CONH-$(CH_2)_n$-NH-CS-NH-peptide or -CONH-$(CH_2)_n$-NHCO-CH_2-peptide with m = 1 to 10 and n = 2 or 3,

or R^3 represents a group below:



R^4 and R^5, independently of one another, stand for a hydrogen atom, a methyl radical or a hydroxylated alkyl radical, such as, e.g., 2-hydroxyethyl, 3-hydroxypropyl, 2,3-dihydroxypropyl, 1,3-dihydroxy-2-propyl, 2,3,4-trihydroxybutyl, 1,3,4-trihydroxy-2-butyl, 2,3,4,5,6-pentahydroxyhexyl,

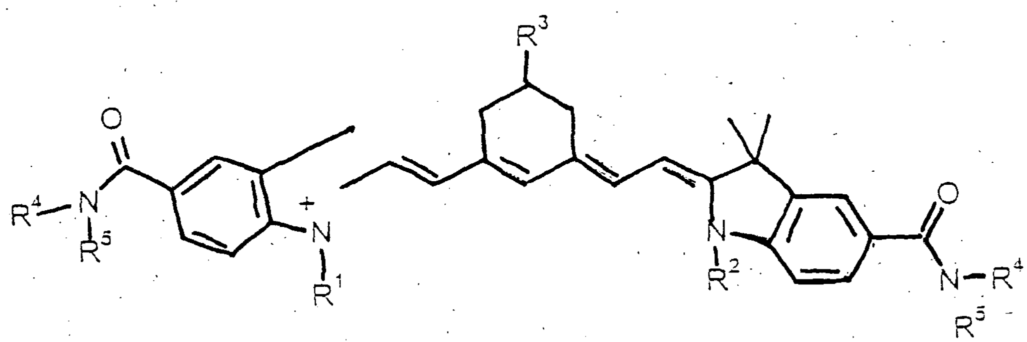
R^6 stands for one of the following groups:

$-(CH_2)_m$-CONH-peptide with m = 0 to 2,

$-(CH_2)_m$-NH-CS-NH-peptide with m = 0 to 2,

and X stands for an oxygen atom or a sulfur atom;

-- dye molecule A^1 and/or A^2 stands for an indotricarbocyanine dye of general formula XVII:



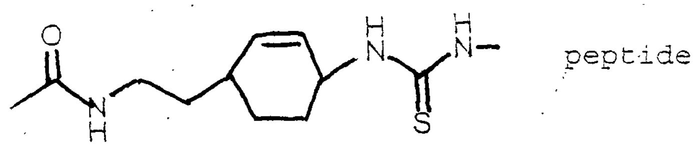
XVII

in which

R^1 and R^2, independently of one another, stand for a 4-sulfobutyl radical or 3-sulfopropyl radical,

R^3 stands for a radical -CONH-peptide, -CONH-$(CH_2)_m$-CONH-peptide, -CONH-$(CH_2)_n$-NH-CS-NH-peptide or -CONH-$(CH_2)_n$-NHCO-CH_2-peptide with m = 1 to 10 and n = 2 or 3,

or R^3 represents a group below:



and R^4 and R^5, independently of one another, stand for a hydrogen atom, a methyl radical or a hydroxylated alkyl radical, such as, e.g., 2-hydroxyethyl, 3-hydroxypropyl, 2,3-dihydroxypropyl, 1,3-dihydroxy-2-propyl, 2,3,4-trihydroxybutyl, 1,3,4-trihydroxy-2-butyl, 2,3,4,5,6-pentahydroxyhexyl.

The use of dye-labeled antibodies for tumor detection is known in the literature (J. Cell. Pharmacol. 3, 141-145, 1992; Cancer Immunol. Immunother. 41, 257-63, 1995; Cancer Research 54, 2643-9, 1994, Biotechnol. Prog. 13, 649-658, 1997).

In contrast, the compounds according to the invention contain as biomolecules low-molecular peptides and peptide derivatives that exhibit the advantages of antibodies, such as a high binding to target structures, without the diagnostic potential being limited by an unfavorable pharmacokinetics (long blood half-lives, allergenic side-effects (immunogenicity)).

Biological and pharmacological requirements on peptide sequences are consequently an adequate plasma stability in the

case of quick build-up in the target tissue and simultaneous quick elimination from the remainder of the body, preferably via the renal excretion route.

It has been found, surprisingly enough, that peptide sequences that contain at least 5 amino acids of the C-terminal side of the VIP-sequence and to which a dye is coupled for fluorescence diagnosis have an imaging in tumor cells that is comparable to the native VIP. Moreover, it has been found that by the incorporation of at least one D-amino acid, or the exchange by at least one D-amino acid, the plasma stability could be considerably increased. In the example of a complete exchange of all L-amino acids for D-amino acids in a VIP-binding dye-peptide conjugate, it was possible to show that the binding properties and the cell imaging are unchanged.

Other especially preferred compounds of general formula I according to the invention are distinguished in that

-- $(X)_m$ stands for the amino acid sequence of the native, naturally occurring, human, vaso-active intestinal peptide corresponding to

HSDAVFTDNYTRLRKQMAVKKYLNSILN (SEQ ID NO: 196)

or for fragments, partial sequences, derivatives or analogs of the vaso-active, intestinal peptide that consists of 5 to 30 amino acids,

-- $(X)_m$ stands for the amino acid sequence of the somatostatin that corresponds to

AGCKNFFWKTFTSC (SEQ ID NO: 9)

or for fragments, partial sequences, derivatives or analogs of the somatostatin that consists of 5 to 20 amino acids,

-- $(X)_m$ stands for the amino acid sequence of the neurotensin that corresponds to

pyroglutamic acid-LYENKPRRPYIL (SEQ ID NO: 10)

or for fragments, partial sequences, derivatives or analogs of the neurotensin that consists of 5 to 20 amino acids,

■ as fragments, partial sequences, derivatives or analogs of the vaso-active intestinal peptide (VIP), the following amino acid sequences are selected:

RLRKQMAVKKYLNSILN (SEQ ID NO: 11)	RLRKQMAVKKYLNSIL (SEQ ID NO: 18)	RLRKQMAVKKYLNSI (SEQ ID NO: 25)
LRKQMAVKKYLNSILN (SEQ ID NO: 12)	LRKQMAVKKYLNSIL (SEQ ID NO: 19)	LRKQMAVKKYLNSI (SEQ ID NO: 26)
RKQMAVKKYLNSILN (SEQ ID NO: 13)	RKQMAVKKYLNSIL (SEQ ID NO: 20)	RKQMAVKKYLNSI (SEQ ID NO: 27)
KQMAVKKYLNSILN (SEQ ID NO: 14)	KQMAVKKYLNSIL (SEQ ID NO: 21)	KQMAVKKYLNSI (SEQ ID NO: 28)
QMAVKKYLNSILN (SEQ ID NO: 15)	QMAVKKYLNSIL (SEQ ID NO: 22)	QMAVKKYLNSI (SEQ ID NO: 29)
MAVKKYLNSILN	MAVKKYLNSIL	MAVKKYLNSI

(SEQ ID NO: 16) (SEQ ID NO: 23) (SEQ ID NO: 30)
AVKKYLNSILN AVKKYLNSIL AVKKYLNSI
(SEQ ID NO: 17) (SEQ ID NO: 24) (SEQ ID NO: 31)

RLRKQMAVKKYLNS RLRKQMAVKKYLN RLRKQMAVKKYL
(SEQ ID NO: 32) (SEQ ID NO: 39) (SEQ ID NO: 46)
LRKQMAVKKYLNS LRKQMAVKKYLN LRKQMAVKKYL
(SEQ ID NO: 33) (SEQ ID NO: 40) (SEQ ID NO: 47)
RKQMAVKKYLNS RKQMAVKKYLN RKQMAVKKYL
(SEQ ID NO: 34) (SEQ ID NO: 41) (SEQ ID NO: 48)
KQMAVKKYLNS KQMAVKKYLN KQMAVKKYL
(SEQ ID NO: 35) (SEQ ID NO: 42) (SEQ ID NO: 49)
QMAVKKYLNS QMAVKKYLN QMAVKKYL
(SEQ ID NO: 36) (SEQ ID NO: 43) (SEQ ID NO: 50)
MAVKKYLNS MAVKKYLN MAVKKYL
(SEQ ID NO: 37) (SEQ ID NO: 44) (SEQ ID NO: 51)
AVKKYLNS AVKKYLN AVKKYL
(SEQ ID NO: 38) (SEQ ID NO: 45) (SEQ ID NO: 52)

- as analogs of the sequence that follows the VIP, the following are selected:

FSDAVFTDNYTRLRKQMAVKKYLNSILN(SEQ ID NO: 53)
ISDAVFTDNYTRLRKQMAVKKYLNSILN(SEQ ID NO: 54)
LSDAVFTDNYTRLRKQMAVKKYLNSILN(SEQ ID NO: 55)
HFDAVFTDNYTRLRKQMAVKKYLNSILN(SEQ ID NO: 56)
HHDAVFTDNYTRLRKQMAVKKYLNSILN(SEQ ID NO: 57)
HIDAVFTDNYTRLRKQMAVKKYLNSILN(SEQ ID NO: 58)
HLDAVFTDNYTRLRKQMAVKKYLNSILN(SEQ ID NO: 59)
HMDAVFTDNYTRLRKQMAVKKYLNSILN(SEQ ID NO: 60)
HQDAVFTDNYTRLRKQMAVKKYLNSILN(SEQ ID NO: 61)
HTDAVFTDNYTRLRKQMAVKKYLNSILN(SEQ ID NO: 62)
HVDAVFTDNYTRLRKQMAVKKYLNSILN(SEQ ID NO: 63)
HWDAVFTDNYTRLRKQMAVKKYLNSILN(SEQ ID NO: 64)
HYDAVFTDNYTRLRKQMAVKKYLNSILN(SEQ ID NO: 65)
HSAAVFTDNYTRLRKQMAVKKYLNSILN(SEQ ID NO: 66)
HSEAVFTDNYTRLRKQMAVKKYLNSILN(SEQ ID NO: 67)
HSFAVFTDNYTRLRKQMAVKKYLNSILN(SEQ ID NO: 68)
HSHAVFTDNYTRLRKQMAVKKYLNSILN(SEQ ID NO: 69)
HSIAVFTDNYTRLRKQMAVKKYLNSILN(SEQ ID NO: 70)
HSLAVFTDNYTRLRKQMAVKKYLNSILN(SEQ ID NO: 71)
HSMAVFTDNYTRLRKQMAVKKYLNSILN(SEQ ID NO: 72)
HSWAVFTDNYTRLRKQMAVKKYLNSILN(SEQ ID NO: 73)
HSDFVFTDNYTRLRKQMAVKKYLNSILN(SEQ ID NO: 74)

HSDGVFTDNYTRLRKQMAVKKYLNSILN(SEQ ID NO: 75)
HSDMVFTDNYTRLRKQMAVKKYLNSILN(SEQ ID NO: 76)
HSDQVFTDNYTRLRKQMAVKKYLNSILN(SEQ ID NO: 77)
HSDSVFTDNYTRLRKQMAVKKYLNSILN(SEQ ID NO: 78)
HSDWVFTDNYTRLRKQMAVKKYLNSILN(SEQ ID NO: 79)
HSDYVFTDNYTRLRKQMAVKKYLNSILN(SEQ ID NO: 80)
HSDAFFTDNYTRLRKQMAVKKYLNSILN(SEQ ID NO: 81)
HSDAIFTDNYTRLRKQMAVKKYLNSILN(SEQ ID NO: 82)
HSDALFTDNYTRLRKQMAVKKYLNSILN(SEQ ID NO: 83)
HSDAMFTDNYTRLRKQMAVKKYLNSILN(SEQ ID NO: 84)
HSDATFTDNYTRLRKQMAVKKYLNSILN(SEQ ID NO: 85)
HSDAWFTDNYTRLRKQMAVKKYLNSILN(SEQ ID NO: 86)
HSDAYFTDNYTRLRKQMAVKKYLNSILN(SEQ ID NO: 87)
HSDAVKTDNYTRLRKQMAVKKYLNSILN(SEQ ID NO: 88)
HSDAVFVDNYTRLRKQMAVKKYLNSILN(SEQ ID NO: 89)
HSDAVFWDNYTRLRKQMAVKKYLNSILN(SEQ ID NO: 90)
HSDAVFTDNWTRLRKQMAVKKYLNSILN(SEQ ID NO: 91)
HSDAVFTDNYTRRRKQMAVKKYLNSILN(SEQ ID NO: 92)
HSDAVFTDNYTRWRKQMAVKKYLNSILN(SEQ ID NO: 93)
HSDAVFTDNYTRLRFQMAVKKYLNSILN(SEQ ID NO: 94)
HSDAVFTDNYTRLRLQMAVKKYLNSILN(SEQ ID NO: 95)
HSDAVFTDNYTRLRMQMAVKKYLNSILN(SEQ ID NO: 96)
HSDAVFTDNYTRLRRQMAVKKYLNSILN(SEQ ID NO: 97)
HSDAVFTDNYTRLRKAMAVKKYLNSILN(SEQ ID NO: 98)
HSDAVFTDNYTRLRKFMAVKKYLNSILN(SEQ ID NO: 99)
HSDAVFTDNYTRLRKIMAVKKYLNSILN(SEQ ID NO: 100)
HSDAVFTDNYTRLRKKMAVKKYLNSILN(SEQ ID NO: 101)
HSDAVFTDNYTRLRKLMAVKKYLNSILN(SEQ ID NO: 102)
HSDAVFTDNYTRLRKMMAVKKYLNSILN(SEQ ID NO: 103)
HSDAVFTDNYTRLRKRMAVKKYLNSILN(SEQ ID NO: 104)
HSDAVFTDNYTRLRKVMAVKKYLNSILN(SEQ ID NO: 105)
HSDAVFTDNYTRLRKWMAVKKYLNSILN(SEQ ID NO: 106)
HSDAVFTDNYTRLRKYMAVKKYLNSILN(SEQ ID NO: 107)
HSDAVFTDNYTRLRKQFAVKKYLNSILN(SEQ ID NO: 108)
HSDAVFTDNYTRLRKQIAVKKYLNSILN(SEQ ID NO: 109)
HSDAVFTDNYTRLRKQKAVKKYLNSILN(SEQ ID NO: 110)
HSDAVFTDNYTRLRKQLAVKKYLNSILN(SEQ ID NO: 111)
HSDAVFTDNYTRLRKQQAVKKYLNSILN(SEQ ID NO: 112)
HSDAVFTDNYTRLRKQRAVKKYLNSILN(SEQ ID NO: 113)
HSDAVFTDNYTRLRKQWAVKKYLNSILN(SEQ ID NO: 114)
HSDAVFTDNYTRLRKQMFVKKYLNSILN(SEQ ID NO: 115)
HSDAVFTDNYTRLRKQMIVKKYLNSILN(SEQ ID NO: 116)
HSDAVFTDNYTRLRKQMKVKKYLNSILN(SEQ ID NO: 117)
HSDAVFTDNYTRLRKQMLVKKYLNSILN(SEQ ID NO: 118)
HSDAVFTDNYTRLRKQMMVKKYLNSILN(SEQ ID NO: 119)
HSDAVFTDNYTRLRKQMQVKKYLNSILN(SEQ ID NO: 120)
HSDAVFTDNYTRLRKQMRVKKYLNSILN(SEQ ID NO: 121)
HSDAVFTDNYTRLRKQMVVKKYLNSILN(SEQ ID NO: 122)
HSDAVFTDNYTRLRKQMWVKKYLNSILN(SEQ ID NO: 123)

HSDAVFTDNYTRLRKQMYVKKYLNSILN(SEQ ID NO: 124)
HSDAVFTDNYTRLRKQMAAKKYLNSILN(SEQ ID NO: 125)
HSDAVFTDNYTRLRKQMAIKKYLNSILN(SEQ ID NO: 126)
HSDAVFTDNYTRLRKQMALKKYLNSILN(SEQ ID NO: 127)
HSDAVFTDNYTRLRKQMAVRKYLNSILN(SEQ ID NO: 128)
HSDAVFTDNYTRLRKQMAVKRYLNSILN(SEQ ID NO: 129)
HSDAVFTDNYTRLRKQMAVKWYLNSILN(SEQ ID NO: 130)
HSDAVFTDNYTRLRKQMAVKKFLNSILN(SEQ ID NO: 131)
HSDAVFTDNYTRLRKQMAVKKWLNSILN(SEQ ID NO: 132)
HSDAVFTDNYTRLRKQMAVKKYLASILN(SEQ ID NO: 133)
HSDAVFTDNYTRLRKQMAVKKYLFSILN(SEQ ID NO: 134)
HSDAVFTDNYTRLRKQMAVKKYLISILN(SEQ ID NO: 135)
HSDAVFTDNYTRLRKQMAVKKYLMSILN(SEQ ID NO: 136)
HSDAVFTDNYTRLRKQMAVKKYLSSILN(SEQ ID NO: 137)
HSDAVFTDNYTRLRKQMAVKKYLVSILN(SEQ ID NO: 138)
HSDAVFTDNYTRLRKQMAVKKYLWSILN(SEQ ID NO: 139)
HSDAVFTDNYTRLRKQMAVKKYLNNILN(SEQ ID NO: 140)
HSDAVFTDNYTRLRKQMAVKKYLNRILN(SEQ ID NO: 141)
HSDAVFTDNYTRLRKQMAVKKYLNWILN(SEQ ID NO: 142)
HSDAVFTDNYTRLRKQMAVKKYLNYILN(SEQ ID NO: 143)
HSDAVFTDNYTRLRKQMAVKKYLNSLLN(SEQ ID NO: 144)
HSDAVFTDNYTRLRKQMAVKKYLNSSLN(SEQ ID NO: 145)
HSDAVFTDNYTRLRKQMAVKKYLNSWLN(SEQ ID NO: 146)
HSDAVFTDNYTRLRKQMAVKKYLNSYLN(SEQ ID NO: 147)
HSDAVFTDNYTRLRKQMAVKKYLNSIFN(SEQ ID NO: 148)
HSDAVFTDNYTRLRKQMAVKKYLNSIIN(SEQ ID NO: 149)
HSDAVFTDNYTRLRKQMAVKKYLNSIWN(SEQ ID NO: 150)
HSDAVFTDNYTRLRKQMAVKKYLNSILW(SEQ ID NO: 151)

-- as an analog of the VIP, a compound is selected according to the following formula: HSDAVFTX^{1}X^{2}Y X^{3}RLRKQMAVK KYLNSILN (SEQ ID NO: 152), in which X^1, X^2 and X^3 can represent any amino acid,

-- 2 to m amino acids, independently of one another, can be exchanged for their respective D-amino acid or for other L- or D-amino acids, whereby m has the above-indicated meaning,

-- at least one of amino acids $(X)_m$, independently of one another, can be exchanged for other amino acids or amino acid derivatives that are not natural,

-- at least one of amino acids $(X)_m$, independently of one another, can be exchanged for other amino acids or amino acid derivatives that are not natural, such as, e.g., naphthalanine, cyclohexylalanine, norleucine, norvaline, α-aminoadipic acid, α-aminobutyric acid, β-alanine, β-cyclohexylalanine, ornithine, sarcosine or δ-hydroxylysine,

-- as an analog of the VIP, a compound is selected according to the following formula: X^{1}SDAVX^{2}TDNX^3 TRLRKQMAVK KX^{4}LNSILN (SEQ ID NO: 153), in which X^1, X^2, X^3 and X^4 can represent amino acids or amino acid derivatives that are not natural, such as,

e.g., naphthalanine, cyclohexylalanine, norleucine, norvaline, α-aminoadipic acid, α-aminobutyric acid, β-alanine, β-cyclohexylalanine, ornithine, sarcosine or δ-hydroxylysine,

-- all amino acids $(X)_m$ are exchanged for their respective D-amino acid,

-- retrosynthetic amino acid sequences are selected as fragments, partial sequences, derivatives or analogs of the vaso-active intestinal peptide,

-- retrosynthetic amino acid sequences, in which 2 to m amino acids are exchanged for the respective D-amino acid, are selected as fragments, partial sequences, derivatives or analogs of the vaso-active, intestinal peptide, whereby m has the above-indicated meaning,

-- the following amino acid sequences are selected as fragments, partial sequences, derivatives or analogs of the vaso-active, intestinal peptide:

rlrkqmavkkylnsiln (SEQ ID NO: 11)	rlrkqmavkkylnsil (SEQ ID NO: 18)	rlrkqmavkkylnsi (SEQ ID NO: 25)
lrkqmavkkylnsiln (SEQ ID NO: 12)	lrkqmavkkylnsil (SEQ ID NO: 19)	lrkqmavkkylnsi (SEQ ID NO: 26)
rkqmavkkylnsiln (SEQ ID NO: 13)	rkqmavkkylnsil (SEQ ID NO: 20)	rkqmavkkylnsi (SEQ ID NO: 27)
kqmavkkylnsiln (SEQ ID NO: 14)	kqmavkkylnsil (SEQ ID NO: 21)	kqmavkkylnsi (SEQ ID NO: 28)
qmavkkylnsiln (SEQ ID NO: 15)	qmavkkylnsil (SEQ ID NO: 22)	qmavkkylnsi (SEQ ID NO: 29)
mavkkylnsiln (SEQ ID NO: 16)	mavkkylnsil (SEQ ID NO: 23)	mavkkylnsi (SEQ ID NO: 30)

avkkylnsiln (SEQ ID NO: 17)	avkkylnsil (SEQ ID NO: 24)	avkkylnsi (SEQ ID NO: 31)

RLRKQMAvKKyLNSILN (SEQ ID NO: 11)	RLRKQMAvKKyLNSIL (SEQ ID NO: 18)	RLRKQMAvKKyLNSI (SEQ ID NO: 25)
LRKQMAvKKyLNSILN (SEQ ID NO: 12)	LRKQMAvKKyLNSIL (SEQ ID NO: 19)	LRKQMAvKKyLNSI (SEQ ID NO: 26)
RKQMAvKKyLNSILN (SEQ ID NO: 13)	RKQMAvKKyLNSIL (SEQ ID NO: 20)	RKQMAvKKyLNSI (SEQ ID NO: 27)
KQMAvKKyLNSILN (SEQ ID NO: 14)	KQMAvKKyLNSIL (SEQ ID NO: 21)	KQMAvKKyLNSI (SEQ ID NO: 28)
QMAvKKyLNSILN (SEQ ID NO: 15)	QMAvKKyLNSIL (SEQ ID NO: 22)	QMAvKKyLNSI (SEQ ID NO: 29)
MAvKKyLNSILN (SEQ ID NO: 16)	MAvKKyLNSIL (SEQ ID NO: 23)	MAvKKyLNSI (SEQ ID NO: 30)
AvKKyLNSILN (SEQ ID NO: 17)	AvKKyLNSIL (SEQ ID NO: 24)	AvKKyLNSI (SEQ ID NO: 31)

- the following amino acid sequences are selected as fragments, partial sequences, derivatives or analogs of the somatostatin:

AGCKNFFwKTFTSC (SEQ ID NO: 9)	AGcKNFFwKTFTSC (SEQ ID NO: 9)
AGCKNFFwKTFTSc (SEQ ID NO: 9)	AGcKNFFwKTFTSc (SEQ ID NO: 9)
CKNFFwKTFTSC (SEQ ID NO: 154)	cKNFFwKTFTSC (SEQ ID NO: 154)
FFYwKVFT (SEQ ID NO: 155)	
fCFwKVCT (SEQ ID NO: 156)	fcFwKVCT (SEQ ID NO: 156)
fCYwKVCT (SEQ ID NO: 157)	fcYwKVCT (SEQ ID NO: 157)
fCFwKTCT (SEQ ID NO: 158)	fcFwKTCT (SEQ ID NO: 158)
fCYwKTCT (SEQ ID NO: 159)	fcYwKTCT (SEQ ID NO: 159)
D-NaI-CYwKVC (SEQ ID NO: 195)	D-NaI-cYwKVC (SEQ ID NO: 195)
fCywK-Abu-C-NaI (SEQ ID NO: 160)	fcywK-Abu-C-NaI (SEQ ID NO: 160)

-- the following amino acid sequences are selected as fragments, partial sequences, derivatives or analogs of the neurotensin:

	pGlu-LYQNKPRRPFIL (SEQ ID NO: 165)	pGlu-LYENKPRRPYI (SEQ ID NO: 171)
pGlu-LYENKPRRPyIL (SEQ ID NO: 10)	pGlu-LYQNKPRRPfIL (SEQ ID NO: 166)	pGlu-LYENKPRRPY (SEQ ID NO: 172)
pGlu-LYQNKPRRPYIL (SEQ ID NO: 161)	pGlu-LYENKPRRPWIL (SEQ ID NO: 167)	pGlu-LYENKPRRP (SEQ ID NO: 173)
pGlu-LYQNKPRRPyIL (SEQ ID NO: 162)	pGlu-LYENKPRRPwIL (SEQ ID NO: 168)	pGlu-LYENKPRR (SEQ ID NO: 174)
pGlu-LYENKPRRPFIL (SEQ ID NO: 163)	pGlu-LYQNKPRRPWIL (SEQ ID NO: 169)	pGlu-LYENKPR (SEQ ID NO: 175)
pGlu-LYENKPRRPfIL (SEQ ID NO: 164)	pGlu-LYQNKPRRPwIL (SEQ ID NO: 170)	pGlu-LYENKP (SEQ ID NO: 176)

NKPRRPYIL (SEQ ID NO: 177)	NKPRRPyIL (SEQ ID NO: 177)	NKPRRPfIL (SEQ ID NO: 181)	NKPRRPwIL (SEQ ID NO: 185)
KPRRPYIL (SEQ ID NO: 178)	KPRRPyIL (SEQ ID NO: 178)	KPRRPfIL (SEQ ID NO: 182)	KPRRPwIL (SEQ ID NO: 186)
PRRPYIL (SEQ ID NO: 179)	PRRPyIL (SEQ ID NO: 179)	PRRPfIL (SEQ ID NO: 183)	PRRPwIL (SEQ ID NO: 187)
RRPYIL (SEQ ID NO: 180)	RRPyIL (SEQ ID NO: 180)	RRPfIL (SEQ ID NO: 184)	RRPwIL (SEQ ID NO: 188)

The terminology of general formula I contains the usual language of amino acid sequences. The N-terminus is always on the left and the C-terminus on the right (unsubstituted according to $H-(X)_m-OH$ or, in the case of an amide, $H-(X)_m-NH_2$). The one-character abbreviations of the amino acids that are used can be looked up in M. Bodanszky, Peptide Chemistry -- A Practical Textbook, 2nd Edition, Springer-Verlag Heidelberg 1993, p. 3. Capital letters mean amino acids with L-configuration (natural amino acids), small letters mean D-amino acids, disulfide bridges

(cyclic peptides) are identified by connecting lines between the corresponding letters (C = cysteine or homocysteine). Retrosynthetically, sequences are designated in which in the synthesis, the sequence of the amino acids is inverted in comparison to a given native sequence, i.e., the synthesis begins with the original N-terminal amino acid and produces the sequence up to the original C-terminal amino acid.

The analogs of the VIP were determined using substitution analysis (see Example 43). Especially preferred analogs of the VIP are the following compounds:

His-Trp-Asp-Ala-Val-Phe-Thr-Asp-Asn-Tyr-Thr-Arg-Leu-Arg-Lys-
Gln-Met-Ala-Val-Lys-Lys-Tyr-Leu-Asn-Ser-Ile-Leu-Asn
(SEQ ID NO: 1)

His-Ser-Asp-Ala-Val-Phe-Thr-Phe-Asn-Tyr-Thr-Arg-Leu-Arg-Lys-
Gln-Met-Ala-Val-Lys-Lys-Tyr-Leu-Asn-Ser-Ile-Leu-Asn
(SEQ ID NO: 2)

His-Ser-Asp-Ala-Val-Phe-Thr-Lys-Asn-Tyr-Thr-Arg-Leu-Arg-Lys-
Gln-Met-Ala-Val-Lys-Lys-Tyr-Leu-Asn-Ser-Ile-Leu-Asn
(SEQ ID NO: 3)

His-Ser-Asp-Ala-Val-Phe-Thr-Gln-Asn-Tyr-Thr-Arg-Leu-Arg-Lys-
Gln-Met-Ala-Val-Lys-Lys-Tyr-Leu-Asn-Ser-Ile-Leu-Asn
(SEQ ID NO: 4)

His-Ser-Asp-Ala-Val-Phe-Thr-Arg-Asn-Tyr-Thr-Arg-Leu-Arg-Lys-Gln-Met-Ala-Val-Lys-Lys-Tyr-Leu-Asn-Ser-Ile-Leu-Asn (SEQ ID NO: 5)

His-Ser-Asp-Ala-Val-Phe-Thr-Trp-Asn-Tyr-Thr-Arg-Leu-Arg-Lys-Gln-Met-Ala-Val-Lys-Lys-Tyr-Leu-Asn-Ser-Ile-Leu-Asn (SEQ ID NO: 6)

His-Ser-Asp-Ala-Val-Phe-Thr-Asp-Asn-Tyr-Arg-Arg-Leu-Arg-Lys-Gln-Met-Ala-Val-Lys-Lys-Tyr-Leu-Asn-Ser-Ile-Leu-Asn (SEQ ID NO: 7)

His-Ser-Asp-Ala-Val-Phe-Thr-Asp-Asn-Tyr-Thr-Arg-Leu-Arg-Lys-Gln-Met-Arg-Val-Lys-Lys-Tyr-Leu-Asn-Ser-Ile-Leu-Asn (SEQ ID NO: 8).

The substances according to the invention have various advantages compared to radiolabeled substances. Fluorescence dyes can often be excited at will for fluorescence emission. No continuous signal is present, which uses as a basis a decomposition with corresponding half-life. Consequently, the time of diagnosis can be selected at will and can be repeated as often as desired and is not limited by the half-life of an isotope. The patient is not exposed to any ionizing radiation,

and the light radiation that is used is harmless in the doses used. The optical detection technique allows the highly sensitive detection of a few photons and is therefore comparable to radiodiagnosis relative to the sensitivity.

A process for diagnosis using near-infrared radiation (NIR radiation) with use of dye biomolecule conjugates was described (WO 96/17628). It has proven especially advantageous in the case of this invention that the peptide conjugates in advantageous yield and high purity can be produced by the process of the automated solid-phase synthesis. It has been found, surprisingly enough, that various carboxyl group-carrying indocyanine compounds can be used as amino acid analogs, and both N-terminal groups and an amino group of the lysine can be coupled to the solid phase (resin). After resin is cleaved off and after chromatographic purification, the dye-peptide conjugates were obtained in purities > 95%. A new coupling process allows the coupling of haloacetyl dyes to the amino acid cysteine or homocysteine, which can replace any amino acid of the native VIP sequence.

The basic problem when using light for fluorescence excitation is the limited penetration depth of the light, which is in the submillimeter range in the VIS, but can be in centimeters in the NIR. With respect to the penetration depth, detection processes are without problems in surface tissue diseases, as well as soft tissues. Subjects of the invention are

therefore mammographic processes, endoscopic processes and intraoperative processes, in which diseased tissue areas are diagnosed with use of the compounds according to the invention by detection of fluorescence or the non-absorbed radiation. A more special subject of the invention is the use of the compounds according to the invention in endoscopic processes, e.g., coloscopy, bronchoscopy, esophageal endoscopy, in which changes in tissue that are near the surface are diagnosed. The use of white light with direct visual evaluation is common in endoscopic diagnosis. The compounds according to the invention contribute to a decisive improvement of the process by the production of a tissue-specific signal, especially in the case of the diagnosis of premature, visually undetectable tissue changes (e.g., dysplasias of the colon).

It has been found that after the dye-coupled compounds according to the invention are atomized in the intestines of rats with chemically induced dysplasias and colon carcinomas, subsequent flushing and the implementation of an endoscopic fluorescence diagnosis (excitation 740 nm, detection above 760 nm) make it possible to detect tissue areas of increased fluorescence in the colon.

The subject of the invention is therefore also a process for endoscopic fluorescence diagnosis, especially of the gastrointestinal tract, with use of the compounds according to the invention. In this case, one or more of the substances are

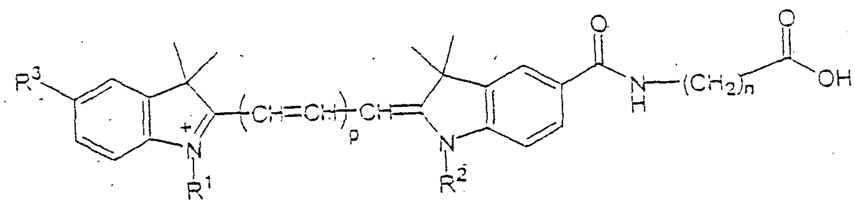
fed to the tissue preferably intravenously, or topically by atomization, and the light from the corresponding spectral range is irradiated for electronic excitation of the dye that is used. The reflected fluorescence radiation or the fluorescence radiation that is emitted by the dye is recorded. Preferred are the methods in which the tissue is irradiated over a large surface, and the fluorescence radiation is indicated with local resolution by imaging with a CCD camera, or the tissue areas that are to be imaged are rastered with a fiber optic light guide, and the signals that are obtained are reacted by computer into a synthetic image. In this case, the fluorescence can be detected and evaluated spectrally and/or phase-selectively as well as in a stationary and/or time-resolved manner. The fluorescence images that are obtained can be produced at the same time as the white light images and are produced over one another in a figure for data evaluation.

The synthesis of the compounds according to the invention is carried out in a way that is similar to the methods that are known in the literature. The peptides are produced in a solid-phase-synthetic manner in polymer resins. Details are known to one skilled in the art. Literature: Peptide Chemistry -- A Practical Textbook (M. Bodanszky), 2nd Edition, Springer-Verlag Heidelberg 1993; Anti-Cancer Drug Design 12, 145-167, 1997; J. Am. Chem. Soc. 117, 11821-2, 1995.

The dyes are produced separately and then, as part of the solid-phase-synthetic production of the peptides, the dyes are coupled to the peptides, and the compounds according to the invention are obtained as highly pure compounds after cleavage from resin and purification. Preferred are those dyes that contain carboxyl groups, which after activation with common reagents are coupled to amino groups of the peptide, especially the ξ-amino group of the lysine or the N-terminal peptide-amino group. In addition, dyes with haloalkyl or haloacetyl radicals are preferred that are coupled to thiol groups of the peptide, especially the amino acid cysteine or homocysteine.

Literature on the synthesis of polymethine dyes: Bioconjugate Chem. 4, 105-111, 1993: Bioconjugate Chem. 7, 356-62, 1996; Bioconjugate Chem. 8, 751-56, 1997; Cytometry 10, 11-19, 1989 and 11, 418-30, 1990; J. Heterocycl. Chem. 33, 1871-6, 1996; J. Org. Chem. 60, 2391-5, 1995; Dyes and Pigments 17, 19-27, 1991, Dyes and Pigments 21, 227-34, 1993; J. Fluoresc. 3, 153-155, 1993; Anal. Biochem. 217, 197-204, 1994; US 4981977; US 5688966; US 5808044; WO 97/42976; WO 97/42978; WO 98/22146; WO 98/26077; EP 0800831.

Especially suitable for a coupling to peptides in the solid phase are dyes that contain precisely one carboxyl group, especially advantageously cyanine dyes of general formula XVIII



XVIII

in which

p stands for 1, 2 or 3,

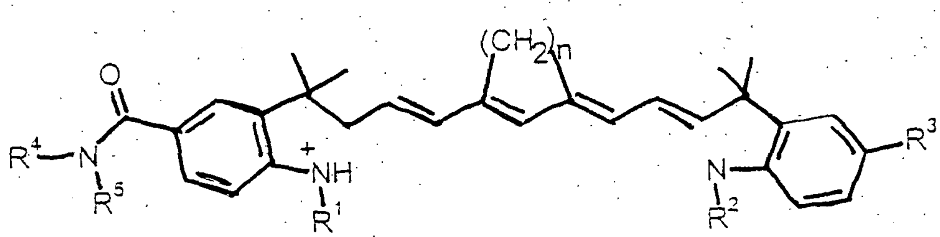
n stands for 1, 2, 3, 4 or 10,

R^1 and R^2, independently of one another, stand for a 4-sulfobutyl, 3-sulfopropyl, 2-sulfoethyl, 3-methyl-3-sulfopropyl, methyl, ethyl or propyl radical, and

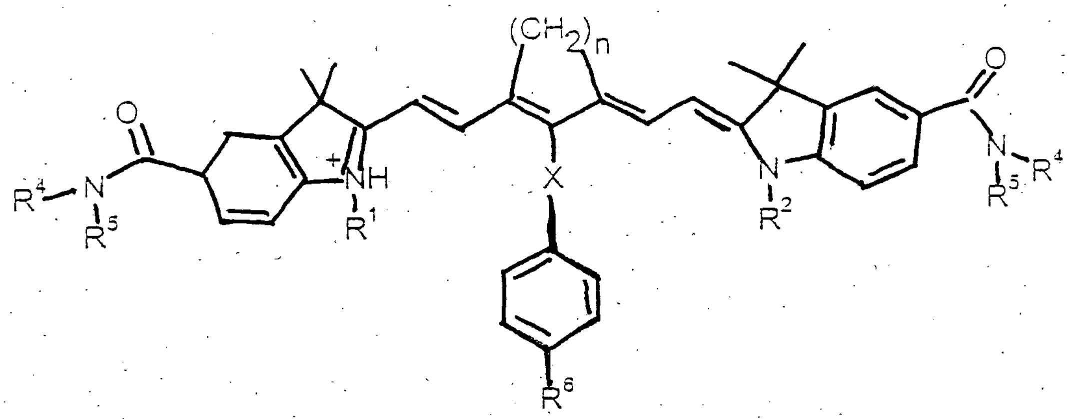
R^3 stands for hydrogen or for a radical $-COOE^1$, $-CONE^1E^2$, $-NHCOE^1$, $-NHCONHE^1$, $-NE^1E^2$, $-OE^1$, $-OSO_3E^1$, $-SO_3E^1$, $-SO_2NHE^1$,

whereby E^1 and E^2, independently of one another, stand for a hydrogen atom or for a methyl, ethyl or a C_3-C_6 alkyl radical, which is interrupted by 0 to 2 oxygen atoms and/or by 0 to 1 carbonyl groups and/or is substituted by 0 to 5 hydroxy groups,

or cyanine dyes of general formula XIX or XX



XIX



XX

in which

n stands for 2 or 3,

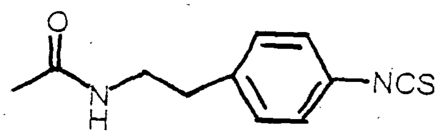
R^1 and R^2, independently of one another, represent a 4-sulfobutyl, 3-sulfopropyl or 2-sulfoethyl radical,

R^3 stands for a -COOH group or one of the following radicals:

-CONH-$(CH_2)_n$-COOH with n = 2 or 3,

-CONH-$(CH_2)_n$-NCS with n = 2 or 3,

$-CONH-(CH_2)_n-NHCO-CH_2-X^1$ with $n = 2$ or 3 and $X^1 = Cl$, Br, I



R^4 and R^5, independently of one another, stand for a hydrogen atom, a methyl radical or a hydroxylated alkyl radical, such as, e.g., 2-hydroxyethyl, 3-hydroxypropyl, 2,3-dihydroxypropyl, 1,3-dihydroxy-2-propyl, 2,3,4-trihydroxybutyl, 1,3,4-trihydroxy-2-butyl, 2,3,4,5,6-pentahydroxyhexyl,

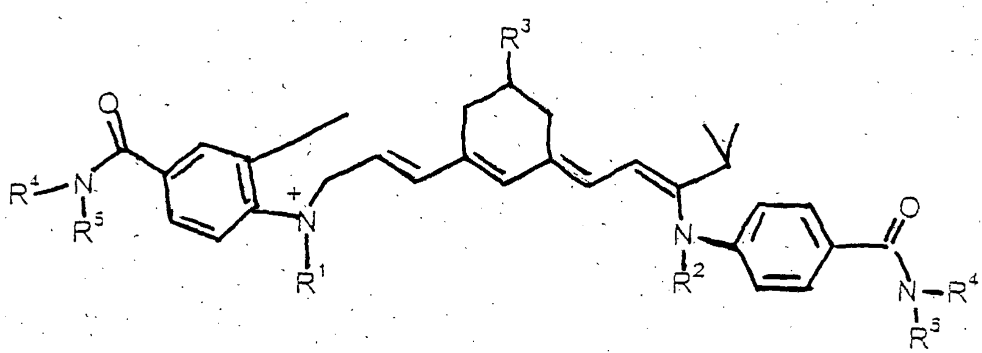
R^6 stands for one of the following groups:

$-(CH_2)_m-COOH$ with $m = 0$ to 2,

$-(CH_2)_m-NCS$ with $m = 0$ to 2,

and X stands for an oxygen atom or a sulfur atom;

or cyanine dyes of general formula XXI



XXI

in which

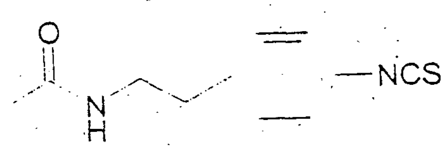
R^1 and R^2, independently of one another, stand for a 4-sulfobutyl-, 3-sulfopropyl or 2-sulfoethyl radical,

R^3 stands for a -COOH group or one of the following radicals:

$-CONH-(CH_2)_n-COOH$ with $n = 2$ or 3,

$-CONH-(CH_2)_n-NCS$ with $n = 2$ or 3,

$-CONH-(CH_2)_n-NHCO-CH_2-X^1$ with $n = 2$ or 3 and $X^1 = Cl$, Br, I



and R^4 and R^5, independently of one another, stand for a hydrogen atom, a methyl radical or a hydroxylated alkyl radical, such as, e.g., 2-hydroxyethyl, 3-hydroxypropyl, 2,3-dihydroxypropyl, 1,3-dihydroxy-2-propyl, 2,3,4-trihydroxybutyl, 1,3,4-trihydroxy-2-butyl, 2,3,4,5,6-pentahydroxyhexyl.

The advantage of only one activatable group, such as, e.g., a carboxyl group, or an already activated group, such as, e.g., an isothiocyanate, a haloalkyl group or a haloacetyl group, consists in the fact that a chemically uniform coupling can be

carried out. The haloacetyl group has the special advantage that a chemically uniform coupling to the mercapto group of the cysteine or homocysteine is carried out. This coupling can be carried out in solution to the unbonded peptide from which protective groups are removed. By the activated groups, a coupling to peptides is possible without secondary reactions occurring. To increase the water solubility, the peptide-dye conjugates in the dye exhibit an increased number of hydroxy groups. By the position of the linker in the indole system of the dye, in addition an adequate hydrophilia by radicals that contain sulfonate groups can be produced in the nitrogen atoms of the indole system. As a result, a structurally uniform coupling reaction can be performed with the peptides (see Examples 4 to 38 and 44 to 49).

Another subject of the invention is an optical diagnostic agent for in-vivo diagnosis of diseased tissue areas, which is distinguished in that it contains at least one compound of general formula I together with common adjuvants and/or vehicles as well as diluents.

The following examples explain the invention:

Examples 1 to 3: **Synthesis of indocyanine dyes 1-3 for solid-phase-synthetic coupling to amino groups (N-terminal or ξ-lysine) of the peptides**

The synthesis is generally carried out starting from 1-(4-sulfobutyl)-2,3,3-trimethyl-3H-indolenine and 1-(4-sulfobutyl)-2,3,3-trimethyl-5-carboxy-3H-indolenine (Cytometry 10, 11-19, 1989, Talanta 39, 505-510, 1992).

Example 1: Synthesis of 1,1'-bis(4-sulfobutyl)indocarbocyanine-5-carboxylic acid, sodium salt (1)

0.8 g (4.0 mmol) of N,N-diphenylformamidine is introduced into 15 ml of acetic acid anhydride and mixed at room temperature in portions with 1.4 g (4.2 mmol) of 1-(4-sulfobutyl)-2,3,3-trimethyl-5-carboxy-3H-indolenine, stirred for 30 minutes at 120°C and then cooled to room temperature with a water bath. Then, 1.2 g (4.1 mmol) of 1-(4-sulfobutyl)-2,3,3-trimethyl-3H-indolenine, 1.2 g (14.6 mmol) of anhydrous sodium acetate, 15 ml of acetic acid anhydride and 6 ml of acetic acid are added. The reaction mixture is heated for 1 hour to 120°C, and the dark red solution is cooled and mixed with 100 ml of ether. The precipitated solid is filtered off. A chromatographic purification on RP-silica gel EUROPREP 60-30 C18, 60A, 20-45 m is carried out (eluant: water/MeOH, step gradient of 0% to 70%

MeOH). Methanol is removed from the product-containing fractions in a rotary evaporator, and the fractions are then freeze-dried, yield: 1.5 g (58%), red lyophilizate.

Example 2:

Synthesis of 1,1'-bis(4-sulfobutyl)indodicarbocyanine-5-carboxylic acid, sodium salt (2)

1.2 g (4.1 mmol) of 1-(4-sulfobutyl)-2,3,3-trimethyl-3H-indolenine and 1.0 g (3.9 mmol) of malonaldehyde-bis-phenylimine hydrochloride are stirred in 15 ml of acetic acid anhydride for 30 minutes at 120°C and then cooled to room temperature with a water bath. Then, 1.4 g (4.2 mmol) of 1-(4-sulfobutyl)-2,3,3-trimethyl-5-carboxy-3H-indolenine, 1.2 g (14.6 mmol) of anhydrous sodium acetate, 15 ml of acetic acid anhydride and 6 ml of acetic acid are added in succession. The reaction mixture is heated for 1 hour to 120°C, the solution that is now blue is cooled and mixed with 100 ml of ether. The working-up and purification are carried out as described in Example 1. Yield: 1.8 g (66%), blue lyophilizate.

Example 3:

Synthesis of 1,1'-bis(4-sulfobutyl)indotricarbocyanine-5-carboxylic acid, sodium salt (3)

1.2 g (4.1 mmol) of 1-(4-sulfobutyl)-2,3,3-trimethyl-3H-indolenine and 1.1 g (3.9 mmol) of glutaconaldehyde-dianil

hydrochloride are stirred in 15 ml of acetic acid anhydride for 30 minutes at 120°C and then cooled to room temperature with a water bath. Then, 1.4 g (4.2 mmol) of 1-(4-sulfobutyl)-2,3,3-trimethyl-5-carboxy-3H-indolenine, 1.2 g (14.6 mmol) of anhydrous sodium acetate, 15 ml of acetic acid anhydride and 6 ml of acetic acid are added. The reaction mixture is heated for 1 hour to 120°C, the now blue solution is cooled and mixed with 100 ml of ether. The working-up and purification are carried out as described in Example 1, yield: 1.8 g (60%), blue lyophilizate.

The structures of the compounds of Examples 1-3 are depicted in Fig. 1.

<u>Examples 4 to 6:</u> **Synthesis of indocyanine dyes 4-6 from 1-3 for solid-phase-synthetic coupling to amino groups (N-terminal or ξ-lysine) of the peptides**

The synthesis is carried out by amidation of dyes 1-3 with β-alanine-t-butylester and acidic cleavage of the t-butylester group.

A solution of 0.5 mmol of dyes 1-3 and 0.1 g (1.0 mmol) of triethylamine in 20 ml of dimethylformamide is mixed at 0°C with 0.5 mmol of TBTU in 10 ml of dimethylformamide, and it is stirred for 15 minutes at 0°C. Then, a solution of 0.11 g (0.6 mmol) of β-alanine-t-butylester-hydrochloride and 0.6 mmol of triethylamine in 5 ml of dimethylformamide is added in drops, and

the reaction mixture is stirred for 2 hours at room temperature. After 100 ml of diethyl ether is added, the precipitated solid is filtered off, dissolved in 20 ml of dichloromethane, mixed with 10 ml of trifluoroacetic acid and stirred for 24 hours at room temperature. The mixture is concentrated by evaporation in a vacuum, and the residue is purified by chromatography and freeze-dried as described in Example 1; yields: 0.21 g (58%) of **4**, 0.29 g (76%) of **5**, 0.28 g (72%) of **6**.

Examples 7 to 9: **Synthesis of indocyanine dyes 7-9 from 1-3 for solid-phase-synthetic coupling to amino groups (N-terminal or ξ-lysine) of the peptides**

The production is carried out analogously to Examples 4-6 with use of 0.1 g (0.6 mmol) of glycine-t-butylester-hydrochloride, yields: 0.25 g (68%) of **4**, 0.30 g (80%) of **5**, 0.32 g (83%) of **6**.

Examples 10 to 12: **Synthesis of indocyanine dyes 10-12 from 1-3 for solid-phase-synthetic coupling to peptides by coupling to thiol groups of cysteine.**

The synthesis is carried out by amidation of dyes 1-3 with 3-aminopropanol and subsequent conversion of the alcohol group into a bromide.

A solution of 0.5 mmol of dyes **1-3** and 0.1 g (1.0 mmol) of triethylamine in 20 ml of dimethylformamide is mixed at 0°C with 0.5 mmol of TBTU in 10 ml of dimethylformamide, and it is stirred for 15 minutes at 0°C. Then, a solution of 850 mg (1.2 mmol) of 3-aminopropanol and 1.2 mmol of triethylamine in 5 ml of dimethylformamide is added in drops, and the reaction mixture is stirred for 6 hours at room temperature. After 100 ml of diethyl ether is added, the precipitated solid is filtered off and purified by chromatography and freeze-dried as described in Example 1.

The reaction to bromides **10-12** is carried out by stirring 0.3 mmol of the intermediate products with 55 mg (0.5 mmol) of N-bromosuccinimide and 130 mg (0.5 mmol) of triphenylphosphine in a mixture of 4 ml of dichloromethane and 4 ml of dimethylformamide for 48 hours at 4°C. By adding 3 ml of ether, the products are precipitated, filtered off and used as crude products in the peptide coupling.

<u>Examples 14-16 and 18-27:</u> **Resin synthesis of peptide conjugates of VIP-receptor-binding peptides and dyes 1-9 and 13.**

a) Solid-phase peptide synthesis: According to the Fmoc strategy and analogously to the standard-Fmoc machine protocol (Pept. Res., 36 (1990) 225), the peptides are synthesized in 50 µmol of TentaGel-Sram resin (Rapp Polymers, Tübingen) with use of

coupling reagent PyBOP (benzotriazol-1-yloxy-tris-pyrrolidinophosphonium hexafluorophosphate) and N-methylmorpholine. The following side-protective groups were used: trityl for Cys, His, Asn and Gln; t-butyl for Asp, Glu, Ser and Thr; t-butyloxycarbonyl for Lys and Trp, and pentamethylchromanosulfonyl for Arg.

The coupling to the ξ-amino group of a lysine (Example 24) is achieved by orthogonal protective group technology. As a lysine component, Fmoc-Lys(Dde-OH) is used, and the peptide is synthesized as described above. After acetylation of the N-terminus, the cleavage of the Dde-protective groups is carried out selectively by 3% hydrazine hydrate.

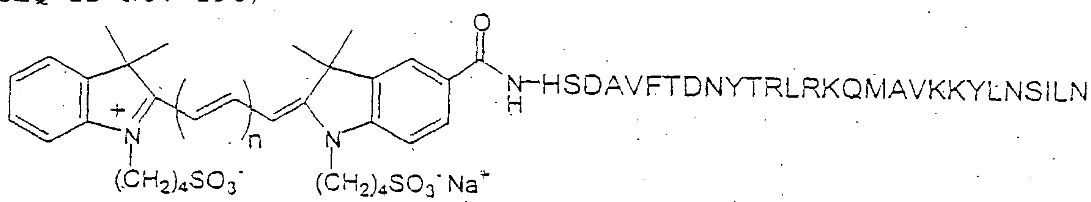
b) **Dye coupling**: The dyes are bonded in the N-terminal position or to lysine in the peptides that are still solid-phase-bonded. In this connection, 75 µmol of the respective dyes **1-9** and **13** (1.5 eq) in 600 µl of dimethylformamide is dissolved and mixed with 83 µmol of TBTU (2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium-tetrafluoroborate) and 150 µmol of N,N-diisopropylethylamine. After 2 minutes, this reaction mixture is added to the respective peptide-carrying resin and allowed to react overnight. Then, the resin is washed 5 times with dimethylformamide and 3 times with dichloromethane and dried in air.

c) **Protective group cleavage and detachment of the dye-peptide-conjugates:** 1.5 ml each of a mixture that consists of 750 mg of phenol, 250 µl of ethanedithiol, 500 µl of thioanisole and 500 µl of water in 10 ml of trifluoroacetic acid are added to the peptide-conjugate-carrying resin and allowed to act for four hours. The dye-peptide conjugates are precipitated with cold t-butylmethyl ether, washed six times with cold diethyl ether, dissolved in 5% acetic acid and freeze-dried. Purity analysis and purification of the conjugates are carried out with use of RP-HPLC in a Vydac-C18 column (gradient: water+0, 05% TFA/acetonitrile, 5% to 60% acetonitrile in 20 minutes, detection: 214 and 750 nm).

The structures of the synthesized dye-peptide conjugates are summarized in the following survey:

Dye Conjugates with VIP-Receptor-Binding Peptides I

Examples 14 to 16: Conjugates of dyes **1-3** with VIP (1-28) (SEQ ID NO: 196)

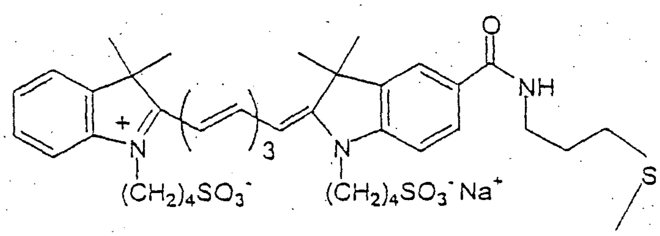


n = 1: Indocarbocyanine-VIP (1-28)-conjugate **14**

n = 2: Indodicarbocyanine-VIP (1-28)-conjugate **15**

n = 3: Indotricarbocyanine-VIP (1-28)-conjugate **16**

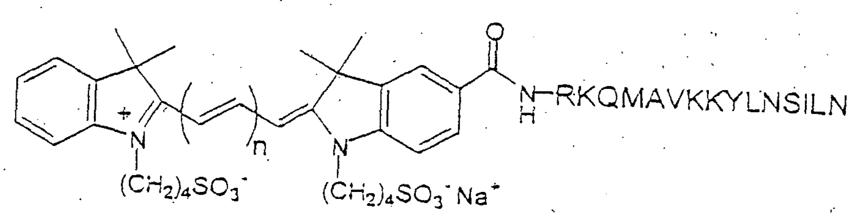
Example 17: Conjugate of Dye **12** with Cys^{17}-VIP (1-28)



HSDAVFTDNYTRLRKQ-Cys-AVKKYLNSILN (SEQ ID NO: 189)

Indotricarbocyanine-Cys^{17}-VIP (1-28)-conjugate **17**

Examples 18 to 20: Conjugates of dyes **1-3** with VIP (14-28) (SEQ ID NO: 13)

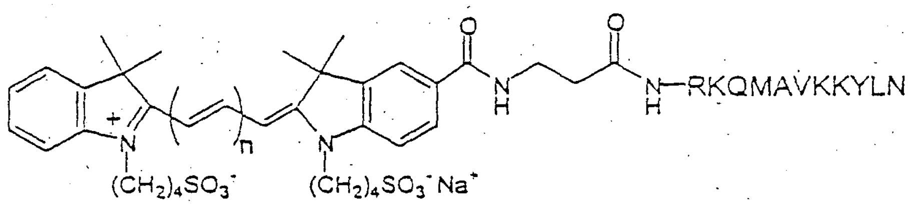


n = 1: Indocarbocyanine-VIP (14-28)-conjugate **18**

n = 2: Indodicarbocyanine-VIP (14-28)-conjugate **19**

n = 3: Indotricarbocyanine-VIP (14-28)-conjugate **20**

Examples 21 to 23: Conjugates of dyes **4-6** with VIP (14-24) (SEQ ID NO: 190)



n = 1: Indocarbocyanine-β-alanine-VIP (14-24)-conjugate **21**

n = 2: Indodicarbocyanine-β-alanine-VIP (14-24)-conjugate **22**

n = 3: Indotricarbocyanine-β-alanine-VIP (14-24)-conjugate **23**

<u>Example 17:</u> **Resin synthesis of peptide conjugates that consist of VIP-receptor-binding peptides and dyes 10-12.**

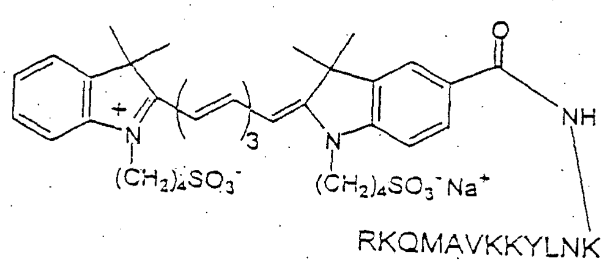
To bind bromine-carrying dyes **10-12** chemoselectively via a thioether bond to the peptides, a cysteine or homocysteine with an orthogonal protective group must be incorporated in the peptide. The solid-phase-peptide synthesis is carried out as described for Examples 14-16/18-27, and the component Fmoc-cys

(Mmt)-OH is used. The monomethoxytrityl group (Mmt) can be cleaved by 1% TFA/5% triisobutylsilane in dichloromethane while obtaining the other side protective groups. In this connection, the resin is incubated three times with 1 ml each of the above solution for 10 minutes. After the resin is washed with dichloromethane (3x), DMF (5x) and ethanol (3x), the resin is incubated for 2 minutes with a 20% cesium carbonate solution (1 ml) and then washed with water (2x), ethanol (2x) and DMF (2x). Then, 75 μmol of respective dye **10-12** (1.5 eq) is dissolved in 600 μl of dimethylformamide and added to the respective resin, and the process is repeated after 30 minutes. Then, the resin is washed five times with dimethylformamide (5x) and with dichloromethane (2x). After the resin is dried in air, the protective group cleavage and detachment of the vehicle are carried out as described above.

The structures of other synthesized dye-peptide conjugates are summarized in the following survey:

Dye Conjugates with VIP-Receptor-Binding Peptides II

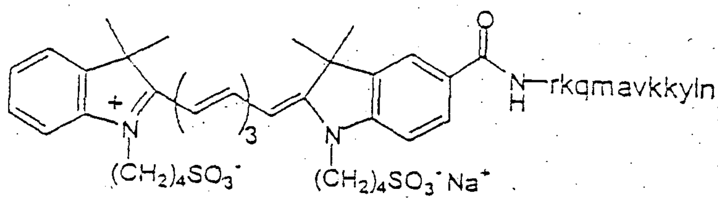
Example 24: Conjugate of dye **6** with Lys^{25}-VIP (14-25) (SEQ ID NO: 191)



(ξ-Amino-indotricarbocyanine)-Lys^{25}-VIP (14-25)-conjugate 24

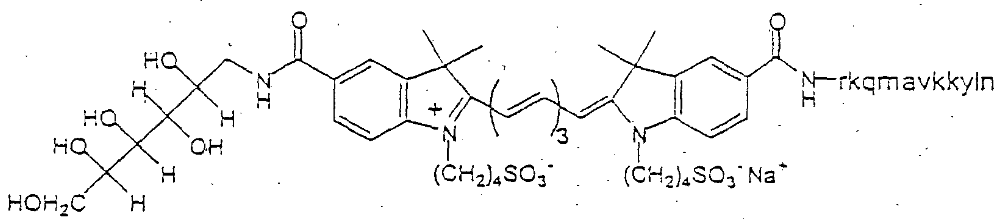
Example 25: Conjugate of dye 3 with D-VIP (14-24)

(SEQ ID NO: 192)



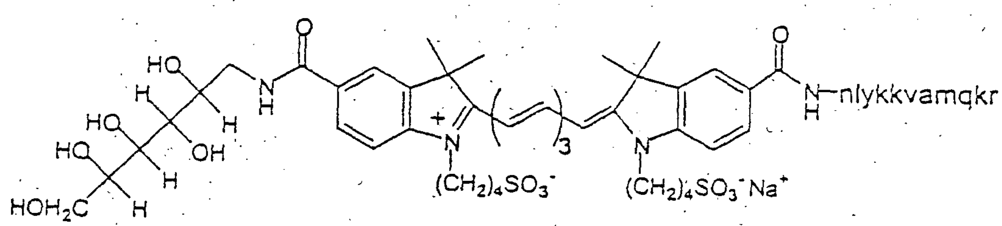
Indotricarbocyanine-D-VIP (14-24)-conjugate 25

Example 26: Conjugate of dye **13** with D-VIP (14-24) (SEQ ID NO: 192)



Indotricarbocyanine-5-carboxylic acid glucamide-D-VIP (14-24)-conjugate **26**

Example 27: Conjugate of dye **13** with retro-D-VIP (14-24) (SEQ ID NO: 193)



Indotricarbocyanine-5-carboxylic acid glucamide-retro-D-VIP (14-24)-conjugate **27**

<u>Examples 28 to 32:</u> **Resin synthesis of peptide conjugates that consist of somatostatin-receptor-binding peptides and dyes 1-9 and 13.**

General Instructions:

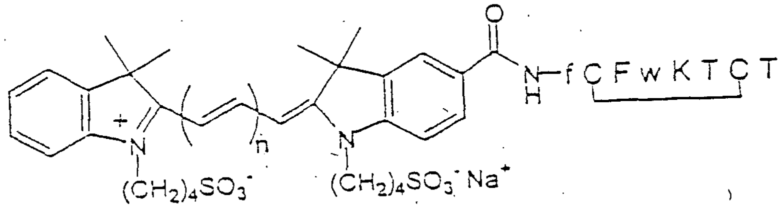
The syntheses were performed on 500 mg of TCP-Thr (But)-fmoc-resin (Pepchem Tübingen Company) with a concentration of 0.49 mmol/g. The peptide is synthesized "step by step" with use of the following temporary protective groups: tert-butyl for Thr and Ser, trityl for Cys and Asp, Boc for Trp and Lys. As a condensation reagent, HBTU is used.

After cleavage of the N-terminal Fmoc-protective group, dyes **1-9, 13** are condensed in a special synthesis step. To this end, 255 mg of resin is suspended in about 2 ml of DMF and mixed with 0.5 mmol of dye, 0.5 mmol of HBTU and 0.17 ml of DIEA. It is stirred for 18 hours at room temperature, the resin is suctioned off, washed with dichloromethane and dried. The cleavage of the dye-peptide conjugate from resin was carried out with 95% trifluoroacetic acid with the addition of triisopropylsilane with subsequent freeze-drying from 10% acetic acid. The crude product is cyclized with use of activated carbon and purified by chromatography (50x300 mm VYDAC RP-18, gradient: water/acetonitrile).

The structures of the synthesized dye-peptide conjugates are summarized in the following survey:

Dye Conjugates with Somatostatin-Receptor-Binding Peptides

Examples 28 to 30: Conjugate of dyes 1-3 with pentetreotide (SEQ ID NO: 158)

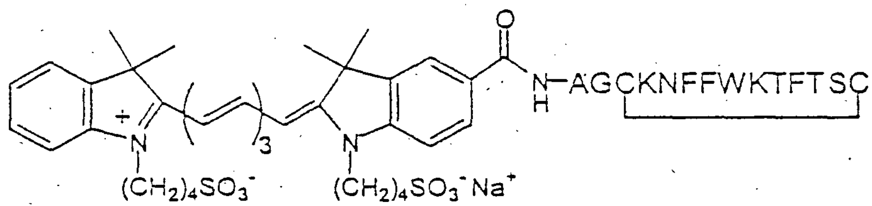


n = 1: Indocarbocyanine-pentetreotide **28**

n = 2: Indodicarbocyanine-pentetreotide **29**

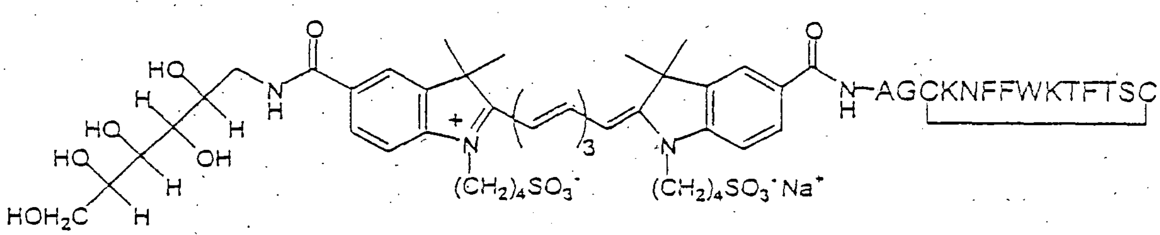
n = 3: Indotricarbocyanine-pentetreotide **30**

Example 31: Conjugate of dye **3** with somatostatin-14 (SEQ ID NO: 9)



Indotricarbocyanine-somatostatin-14-conjugate **31**

Example 32: Conjugate of dye **13** with somatostatin-14 (SEQ ID NO: 9)



Indotricarbocyanine-5-carboxylic acid-glucamide-somatostatin-14-conjugate **32**

Examples 33 to 38: **Synthesis of peptide conjugates from neurotensin peptides and dye 3.**

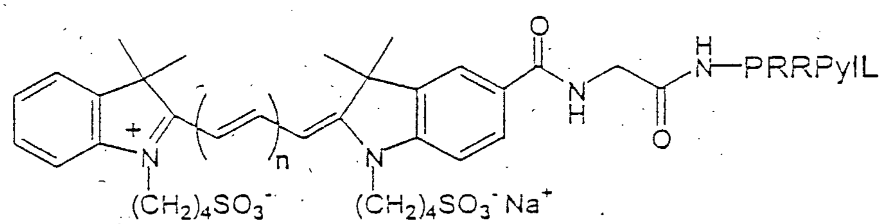
The synthesis of substances is carried out analogously to the general protocols that are described for Examples 14-27.

The structures of the synthesized dye-peptide conjugates are summarized in the following survey:

Dye Conjugates with Neurotensin-Receptor-Binding Peptides

Examples 33 to 35: Conjugate of dyes 7-9 with D-Tyr^{11}-neurotensin (7-13)

(SEQ ID NO: 179)



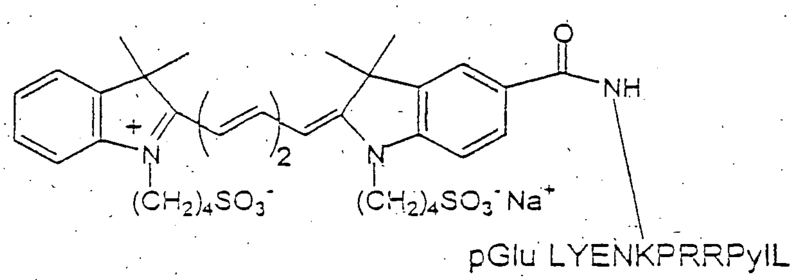
n = 1: Indocarbocyanine-D-Tyr^{11}-neurotensin (7-13)-conjugate **33**

n = 2: Indodicarbocyanine-D-Tyr^{11}-neurotensin (7-13)-conjugate **34**

n = 3: Indotricarbocyanine-D-Tyr^{11}-neurotensin (7-13)-conjugate **35**

Example 36: Conjugate of dye **2** with D-Tyr^{11}-neurotensin

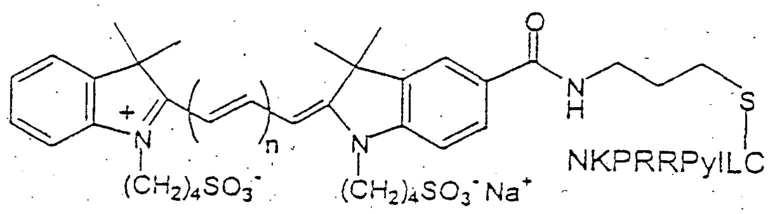
(SEQ ID NO: 10)



(ξ-amino-Lys^6-indo-

dicarbocyanine)-D-
Tyr^{11}-neurotensin-
conjugate **36**

Examples 37 to 38: Conjugate of dyes **10-11** with D-Tyr^{11}-neurotensin (5-13)-Cys
(SEQ ID NO: 194)



n = 1: Indocarbocyanine-D-Tyr^{11}-neurotensin (5-13)-Cys-conjugate **37**

n = 2: Indodicarbocyanine-D-Tyr^{11}-neurotensin (5-13)-Cys-conjugate **38**

<u>Example 39:</u> **Absorption and fluorescence properties of the synthesized dye-peptide conjugates**

Absorption maxima and extinction coefficients were determined in PBS and in bovine plasma (Perkin Elmer Lambda 2). Fluorescence emission spectra were obtained in PBS by excitation on the short-wave side (about 40 nm from the absorption maximum) (SPEX Fluorolog, R928 PMT).

The absorption and fluorescence data are summarized in Fig. 4. In Fig. 5, typical absorption and fluorescence emission spectra are indicated by way of example.

Example 40: **Determination of the cell imaging using fluorescence microscopy**

The binding and imaging of the compounds according to the invention were studied in vitro in human tumor cells, and the receptors for vaso-active intestinal peptide and/or somatostatin and/or neurotensin were expressed. To this end, 5×10^5 tumor cells were incubated in 1.5 ml of medium, which contained the test substance. Different concentrations of the substances (10 nM - 10 µM) were used, and the incubation period varied (1 minute-24 hours). After the incubation, the cells were set, and microscopic preparations were produced. The evaluation was carried out on a Zeiss Axiovert 135-fluorescence microscope, which was equipped with a Cy7- (Exciter HQ 710/70 nm, emitter 810/90 nm, beam splitter 750 nm LP), Cy5- (exciter 575-625 nm, emitter 660-710 nm BP, beam splitter 645 nm) and Cy3-filter set (exciter 546/12 nm, emitter 590 nm LP, beam splitter 580 nm). From all preparations, white light and fluorescence images were recorded with a CCD-camera (Visitron RTE/CCD-576) and digitally stored.

Selected results are described below:

Microscopic white light and fluorescence images (Cy7-filter set) of HT29 cells were set up after 30 minutes of incubation with 10 μM indotricarbocyanine-VIP (1-28)-conjugate **16**. In the fluorescence image, a largely homogeneous fluorescence dispersed over the cells was detected. In addition, areas of increased signals were visible, which can be associated with vesicular compartments. The cells in the fluorescence image correlate in their spatial expansion with the white light image.

With the following compounds, white light and fluorescence images (Cy7-filter set) were obtained by analogous means:

Indotricarbocyanine-VIP (14-28)-conjugate **20**, 10 μM, HT29-cells. Fluorescence dispersed homogeneously over the cells, good correlation with the white light image.

Indotricarbocyanine-retro-D-VIP (24-14) **25**, 10 μM, HT29-cells. Fluorescence dispersed homogeneously over the cells, good correlation with the white light image.

Indotricarbocyanine-pentetreotide conjugate **30**, 10 μM, RIN38-VIP1-cells. Fluorescence areas with a vesicular pattern in the membrane area of the cell, good correlation with the white light image.

In addition, fluorescence images were obtained by analogous means with the following compounds: (-amino-indotricarbocyanine)-Lys^{25}-VIP (14-25)-conjugate **24**, 10 μM,

RIN38-VIP1-cells, Cy7-filter set. The fluorescence image shows a homogeneous, intracellular near-nucleus fluorescence.

Indodicarbocyanine-VIP (1-28)-conjugate **15**, 10 μM, RIN38-VIP1-cells, Cy5-filter set. The fluorescence image shows intracellular fluorescence areas with a vesicular pattern in the membrane area of the cell.

Indocarbocyanine-VIP (1-28)-conjugate **14**, 10 μM, RIN38-VIP1-cells, Cy3-filter set. The fluorescence image shows intracellular fluorescence areas with a vesicular pattern in the membrane area of the cell.

<u>Example 41:</u> **Study of the tumor concentration using in-vivo-fluorescence imaging in tumor-carrying mice**

The imaging properties of the compounds according to the invention were studied in vivo after injection into tumor-carrying nude mice. To this end, 0.1 µmol/kg to 2 µmol/kg of the substance was administered intravenously, and the concentration in the tumor region was observed in a period from 0 to 48 hours. The fluorescence of the substances was excited by irradiation of animals with near-infrared light of wavelength 640 nm (indodicarbocyanines) or 740 nm (indotricarbocyanines), which was produced with an Nd:YAG laser. The fluorescence radiation was detected at a wavelength of > 700 nm or > 800 nm by an intensified CCD-camera, and the fluorescence images were stored digitally.

Selected results are described below:

From a tumor-carrying nude mouse (HT29-tumor in the right rear flank), whole-body-fluorescence images were recorded before and 1 hour after administration of 0.1 µmol/kg of indotricarbocyanine-VIP (14-28)-conjugate 20. The fluorescence intensity before administration is negligible (low autofluorescence). One hour after administration, a signal with approximately a 2-fold increase in intensity resulted in the tumor relative to the contralateral flank in fluorescence

emission, whereby such emission is otherwise dispersed homogeneously over the remainder of the body.

From a tumor-carrying nude mouse (RIN38-SSTR2-tumor in the right rear flank), whole-body-fluorescence images were recorded before and one hour after administration of 0.1 μmol/kg of indotricarbocyanine-pentetreotide conjugate **30**. The fluorescence intensity before administration is negligible (low autofluorescence). One hour after administration, a signal with approximately a 3-fold increase in intensity resulted in the tumor relative to the contralateral flank in fluorescence emission, whereby such emission is otherwise dispersed homogeneously over the remainder of the body.

From a tumor-carrying nude mouse (HT29 tumor in the right rear flank), whole-body-fluorescence images were recorded before and one hour after administration of 0.1 μmol/kg of (ξ-amino-indotricarbocyanine)-Lys^{25}-VIP (14-25)-conjugate **24**. The fluorescence intensity before administration is negligible (low autofluorescence). One hour after administration, a signal with approximately a 1.5-fold increase in intensity resulted in the tumor relative to the contralateral flank. In addition, an increased fluorescence signal in the kidneys was detected.

From a tumor-carrying nude mouse (HT29-tumor in the right rear flank), whole-body-fluorescence images were recorded before and five minutes after administration of 0.2 μmol/kg of

indodicarbocyanine-VIP (1-28)-conjugate 15. The fluorescence intensity before administration is negligible (low autofluorescence). One hour after administration, a signal with approximately a 1.4-fold increase in intensity resulted in the tumor relative to the contralateral flank. In addition, an increased fluorescence signal was detected in the kidneys.

Example 42: **Study of the stability of dye-peptide conjugates in bovine plasma**

The chemical stability of the compounds according to the invention in plasma was studied in vitro using HPLC based on time. To this end, 1 mM solutions of peptides in PBS in bovine blood plasma (Graeber Company, frozen, for heparin analysis) was pipetted while obtaining a concentration of 30 μM, and the solutions were incubated at 37°C.

At various times (0.5; 1; 2; 4; 6; 24 hours), the working-up of the samples was carried out by 1 ml of the plasma solution being mixed with 1 ml of MeOH, and the precipitated proteins being centrifuged off.

The analysis of the supernatant was carried out using HPLC by determining the content at 750 nm relative to the content after 1 minute of incubation at 0°C (control).

HPLC: Beckmann, diode array detector TIDAS (J & M Company), 350-1000 nm;

Column: Chromasil 5 μ, 250 mm x 4.5 mm

Mobile solvent: A: 90% H_2O (+0.5% TFA)/10% MeOH

B: 10% H_2O (+0.5% TFA)/90% MeOH

Gradient: 10% B to 100% B within 20 minutes

The examples are summarized in Fig. 6.

Example 43: **Substitution analysis of VIP using spot synthesis**

1. Synthesis of peptides on cellulose

The synthesis of peptides on cellulose (spot synthesis) was published in 1988 for the first time by R. Frank and R. Döring and described in detail in 1992 by R. Frank[1]. Here, the method[2] established in the AG Schneider-Mergener was used.

The cellulose membrane was chemically modified to provide suitable anchor functions for the subsequent peptide synthesis. In this case, a mercapto group[4] was inserted into an amino-functionalized cellulose membrane (CAPE membrane)[3]. The first amino acid in the form of a bromopropyl ester could be coupled to this mercapto group. Then, all amino acids of the peptide were successively built up according to the Fmoc strategy. At the end, the indodicarbocyanine solid was bonded in the N-terminal position to the peptide, and then all side protective groups were cleaved off.

To be able to study the peptides for receptor binding, the peptides had to be cleaved off from the cellulose. In this respect, a method was developed in which it has been possible for the first time to cleave off peptides with an authentic C-terminus from the cellulose.

1a. Modification of the cellulose membrane

- A 20 x 30 cm cellulose membrane (Whatman 50) was incubated for 2 minutes with methanol/1.2% perchloroacetic acid and then freeze-dried.
- After three hours of incubation with 10% epibromohydrin in dioxane/1.2% perchloroacetic acid, it was reacted for 30 minutes with methanol and then washed twice with methanol.
- Then, it was washed three times with dimethylformamide (DMF) and incubated overnight with 50% 1,3-diaminopropane (v/v) in DMF. Then, it was washed as follows: 3 x DMF, 2 x ethanol, 2 x distilled water, 2 x ethanol, 15 minutes with 5 M sodium methanolate, 3 x methanol, 4 x distilled water, 3 x ethanol, 1 x diethyl ether.

1b. Definition of spots

- For the spot definition, 1.3 µl of 0.6 M Fmoc-ß-alanine-Opfp solution with the auto-spot robot 222 XL (Abimed, Langenfeld) was doubly-pipetted at a reaction time of 15 minutes to certain points on the cellulose membrane.
- The membrane was acetylated for 2 minutes with 2% acetic anhydride solution and for 30 minutes with 20% acetic anhydride/10% diisopropylethylamine.

- For the cleavage of the Fmoc-protective group, the membrane was washed 3 x with DMF, incubated 2 x for 10 minutes with 20% piperidine solution, and washed 5 x with DMF and 1 x with ethanol. The free amino groups could be made visible with bromophenolyl blue. After repeated washing with ethanol, the membrane was dried.

1c. Coupling of the Mmt-mercaptopropionic acid and the bromo-propylester

- 0.6 M mercaptopropionic acid was double-pipetted at a reaction time of 15 minutes to the defined spots. Then, it was washed 3 x with DMF and 3 x with dichloromethane (DCM).
- The cleavage of the Mmt-protective group was carried out with incubation for 2 minutes with 10% dichloroacetic acid/0.5% trifluoroacetic acid and 3 x for 5 minutes with 10% dichloroacetic acid/0.5% trifluoroacetic acid/5% triisobutylsilane. The following washing steps were performed: 1 x DCM, 2 x ethanol, 1 x distilled water, 1-2 minutes with 10% cesium carbonate, 1 x distilled water, 2 x ethanol, 1 x diethyl ether
- The respective Fmoc-bromopropyl-amino acid ester was coupled 3 x at a concentration of 0.6 M and a reaction

time of 15 minutes. The cleavage of the Fmoc-protective group was performed as in 1b.

1d. Coupling of the amino acids

- The peptides were built up to form the spots by repeated pipetting of 0.6 M amino acid solutions in N-methylpyrrolidone and subsequent cleavage of the Fmoc-protective groups.

1e. Coupling of the indodicarbocyanine dye

- An 0.3 M solution of the indodicarbocyanine dye was activated with 0.3 M TBTU and 0.6 M diisopropylethylamine, and it was pipetted 4 x at a reaction time of 15 minutes to the spots.

1f. Cleavage of the side protective groups

- The cleavage of the side protective groups was carried out by subsequent treatment of the membrane with 90% trifluoroacetic acid/3% triisobutylsilane/2% distilled water/1% phenol for 30 minutes and with 50% trifluoroacetic acid/3% triisobutylsilane/2% distilled water/1% phenol for 2.5 hours. Then, it was washed 4 x with dichloromethane, 3 x with DMF and 1 x with ethanol.

2. <u>Cleavage of the peptides from the cellulose-membrane</u>

- The spots were punched out and washed with methanol. For the cleavage, it was incubated for 30 minutes with 70 mM of sodium methanolate in methanol. It was possible to correct the pH by adding 37% hydrochloric acid. Then, the peptides were dried in a speed-vac.
- After drying, the peptides were taken up in distilled water and analyzed using Reversed-Phase-HPLC and MALDI-TOF.

3. Cell assay and continuous-flow cytometry

- The concentration of the VIP derivatives was determined photometrically via the dye. In the cell assay, the peptides were used with a final concentration of 150 mM. In this case, 1 x 10^5 RIN38 (VAPC1) cells were incubated with the VIP derivatives for one hour at 37°C in a binding buffer (50 mM of tris/HCL, pH 7.5, 5 nM of $MgCl_2$, 1 mM of $CaCl_2$, 100 mM of NaCl, 4% BSA).
- Then, the cells were washed 2 x with PBS, moved into the FACS-tube and centrifuged for 5 minutes at 377 g. The cell pellet was measured resuspended in 300 µl of Cellfix in the FACS-Calibur (Becton Dickinson) with an FL4-lens system.

4. Evaluation

- The fluorescence intensities of the native, naturally occurring, human VIP-peptides measured in the continuous-flow cytometry were set at 100%. The standard deviation of 28 native VIP-peptides was 11%. The other VIP-derivatives were adapted to this 100%.

Fig. 7 shows relative fluorescence intensities of RIN38 VPAC1 cells after incubation in the presence of 150 nM of the dye-labeled peptides for 1 hour at 37°C. Data in percentage relative to the native peptide of the respective series.

Literature for spot synthesis:

1. Frank, R. (1992) Spot Synthesis: An Easy Technique for the Positionally Addressable, Parallel Chemical Synthesis on a Membrane Support. **Tetrahedron 48**, 9217-9232

2. Kramer, A.; Schneider-Mergener, J. (1998) Synthesis and Screening of Peptide Libraries on Continuous Cellulose Membrane Supports. **Methods in Molecular Biology** 87, 25-39

3. Volkmer-Engert, R.; Hoffman, B.; Schneider-Mergener, J. (1997) **Tetrahedron Lett.** 38, 1029-1032

4. Licha, K.; Bhargava, S.; Rheinländer, C.; Becker, A.; Schneider-Mergener, J.; Volkmer-Engert, R. (in press) Highly Parallel Nano-Synthesis of Cleavable Peptide-Dye Conjugates on Cellulose Membranes. **Tetrahedron Lett.**

Example 44

a) 5-N-(2,3-Dihydroxypropyl)aminocarbonyl-1-(4-sulfobutyl)-2,3,3-trimethyl(3H)indolenine

0.9 g (2.6 mmol) of 5-carboxy-1-(4-sulfobutyl)-2,3,3-trimethyl(3H)indolenine (Anal. Biochem. 217, 197, 1994) is introduced into 30 ml of absolute N,N-dimethylformamide and 3 ml of pyridine and mixed with 1.35 g (5.3 mmol) of disuccinimidyl carbonate. After three hours, 0.965 g (10.6 mmol) of 2,3-dihydroxypropylamine is added. It is stirred overnight at room

temperature, the batch is evaporated to the dry state, and the residue is absorptively precipitated with diethyl ether. The solid is suctioned off and chromatographed on RP-material for purification.

Yield: 0.82 g (76% of theory)

Analysis (relative to solventless substance):

Cld: C 55.32 H 6.84 N 6.79 S 7.77 O 23.27

Fnd: C 55.39 H 6.95 N 6.57 S 7.58

b) 4-[2-[4-Chloro-7-[5-N-(dihydroxypropyl)aminocarbonyl-3,3-dimethyl-1-(4-sulfonatobutyl)indolin-2-ylidene]-3,5-(propane-1,3-diyl)-1,3,5-heptatrien-1-yl]-5-N-(dihydroxypropyl)aminocarbonyl-3,3-dimethyl(3H)-indolio]-butanesulfonate, sodium salt

A solution of 360 mg (1 mmol) of N-[5-anilino-3-chloro-2,4-(propane-1,3-diyl)-2,4-pentadien-1-ylidene]anilinium-chloride, 825 mg (2 mmol) of 5-N-(2,3-dihydroxypropyl)aminocarbonyl-1-(4-sulfobutyl)-2,3,3-trimethyl(3H)indolenine (Example 44a) and 330 mg (4 mmol) of anhydrous sodium acetate in 30 ml of ethanol are refluxed for two hours under argon. Then, the ethanol is distilled off, and the residue is purified by chromatography.

Yield: 0.58 g (59% of theory)

Analysis (relative to solventless substance):

Cld: C 56.17 H 6.15 Cl 3.60 N 6.79 S 7.77 O 23.27 Na 2.34

Fnd: C 55.99 H 6.30 Cl 3.41 N 6.87 S 7.64 Na 2.17

c) 4-[2-[4-(4-(2-Carboxyethyl)phenyloxy)-7-[5-N-(dihydroxypropyl)aminocarbonyl-3,3-dimethyl-1-(4-sulfonatobutyl)indolin-2-ylidene]-3,5-(propane-1,3-diyl)-1,3,5-heptatrien-1-yl]-5-N-(dihydroxypropyl)aminocarbonyl-3,3-dimethyl(3H)indolio]butanesulfonate, sodium salt, N-hydroxysuccinimide ester

225 mg (1.4 mmol) of 3-(4-hydroxyphenyl)propionic acid is dissolved in 10 ml of dry N,N-dimethylformamide under a cover gas and mixed with 65 mg (2.7 mmol) of sodium hydride (60% in oil). After 30 minutes, 138 mg (0.14 mmol) of 4-[2-[4-chloro-7-[5-N-(dihydroxypropyl)aminocarbonyl-3,3-dimethyl-1-(4-sulfonatobutyl)indolin-2-ylidene]-3,5-(propane-1,3-diyl)-1,3,5-heptatrien-1-yl]-5-N-(dihydroxypropyl)aminocarbonyl-3,3-dimethyl(3H)indolio]butanesulfonate, sodium salt (Example 44b), is added, and it is stirred for another 30 minutes. Then, the reaction mixture is quenched with dry ice and evaporated to the dry state. The residue is purified via a preparative HPLC. For production of the active ester, 14 mg (120 µmol) of N-hydroxysuccinimide and 2 mg (2.4 µmol) of the carboxylic acid are dissolved in 200 µl of N,N-dimethylformamide. After ten minutes, 24 mg (120 µmol) of dicyclohexylcarbodiimide is added, and it is stirred overnight at room temperature. The active ester is used without further purification in the next step.

Analogously to the synthesis according to Examples 14-16 and 18-27a (solid-phase peptide synthesis), the VIP-analog HSDAVFWDNY TRLRKQMAVK KYLNSILN(SEQ ID NO: 90) is synthesized in the solid phase. The dye 4-[2-[4-(4-(2-carboxyethyl)-phenyloxy)-7-[5-N-(dihydroxypropyl)aminocarbonyl-3,3-dimethyl-1-(4-sulfonatobutyl)indolin-2-ylidene]-3,5-(propane-1,3-diyl)-1,3,5-heptatrien-1-yl]-5-N-(dihydroxypropyl)aminocarbonyl-3,3-dimethyl(3H)indolio]-butanesulfonate, sodium salt, is coupled to the peptide according to Examples 14-16 and 18-27b (dye coupling), and this conjugate is isolated and purified according to Examples 14-16 and 18-27c (protective group cleavage and detachment of the dye-peptide conjugates).

d) 4-[2-[4-(4-(2-Isothiocyanatoethyl)phenyloxy)-7-[5-N-(dihydroxypropyl)aminocarbonyl-3,3-dimethyl-1-(4-sulfonatobutyl)indolin-2-ylidene]-3,5-(propane-1,3-diyl)-1,3,5-heptatrien-1-yl]-5-N-(dihydroxypropyl)aminocarbonyl-3,3-dimethyl(3H)indolio]butanesulfonate, sodium salt

116 mg (0.6 mmol) of 2-(4-hydroxyphenyl)ethylisothiocyanate in 2 ml of N,N-dimethylformamide is added to a suspension of 28 mg (0.6 mmol) of sodium hydride (60% in oil) in 4 ml of anhydrous N,N-dimethylformamide at 0°C. After 30 minutes, the solution that is thus produced is added to 138 mg (0.14 mmol) of 4-[2-[4-chloro-7-[5-N-(dihydroxypropyl)aminocarbonyl-5-N-(dihydroxypropyl)aminocarbonyl-3,3-dimethyl-1-(4-

sulfonatobutyl)indolin-2-ylidene]-3,5-(propane-1,3-diyl)-1,3,5-heptatrien-1-yl]-3,3-dimethyl(3H)indolio]butanesulfonate, sodium salt (Example 44b). It is stirred overnight at room temperature and then quenched with dry ice. It is evaporated to the dry state in a rotary evaporator, and the residue is purified via preparative HPLC.

Yield: 85 mg (54% of theory)

Analysis: (relative to solventless substance):

Cld:	C 58.65	H 6.09	N 6.22	S 8.54	O 18.47	Na 2.04
Fnd:	C 58.53	H 6.17	N 6.11	S 8.63		Na 1.83

Analogously to the synthesis according to Examples 14-16 and 18-27a (solid-phase peptide synthesis), the VIP-analog HSDAVFTDNY TRLRFQMAVK KYLNSILN(SEQ ID NO: 94) is synthesized in the solid phase. The dye 4-[2-[4-(4-(2-isothiocyanatoethyl)-phenyloxy)-7-[5-N-(dihydroxypropyl)aminocarbonyl-3,3-dimethyl-1-(4-sulfonatobutyl)indolin-2-ylidene]-3,5-(propane-1,3-diyl)-1,3,5-heptatrien-1-yl]-5-N-(dihydroxypropyl)aminocarbonyl-3,3-dimethyl(3H)indolio]-butanesulfonate, sodium salt, is coupled to the peptide in the N-terminal position, and this conjugate is isolated and purified according to Examples 14-16 and 18-27c (protective group cleavage and detachment of the dye-peptide conjugates).

Analogously, other symmetrically hydrophilic dyes can be built up from the following components:

Hydrophilic indolenine derivatives with hydroxyalkyl substituents: (produced according to Example 44a)

a) 5-N-(2,3-Dihydroxypropyl)-N-methylaminocarbonyl-1-(4-sulfobutyl)-2,3,3-trimethyl(3H)indolenine

b) 5-N-(Hydroxyethyl)-aminocarbonyl-1-(4-sulfobutyl)-2,3,3-trimethyl(3H)indolenine

c) 5-N-(2,3-Dihydroxypropyl)-N-(hydroxyethyl)-aminocarbonyl-1-(4-sulfobutyl)-2,3,3-trimethyl(3H)indolenine

d) 5-N,N-(bis-Hydroxyethyl)-aminocarbonyl-1-(4-sulfobutyl)-2,3,3-trimethyl(3H)indolenine

e) 5-N-(2,3,4,5,6-Pentahydroxyhexyl)-aminocarbonyl-1-(4-sulfobutyl)-2,3,3-trimethyl(3H)indolenine

f) 5-N-(1,3,4-Trihydroxybut-2-yl)-N-methylaminocarbonyl-1-(4-sulfobutyl)-2,3,3-trimethyl(3H)indolenine

Example 45

a) 5-N-(2,3,4,5,6-Pentahydroxyhexyl)aminocarbonyl-1-(4-sulfobutyl)-2,3,3-trimethyl(3H)indolenine

0.6 g (1.8 mmol) of 5-carboxy-1-(4-sulfobutyl)-2,3,3-trimethyl(3H)indolenine (Anal. Biochem. 217, 197, 1994) is introduced into 20 ml of absolute N,N-dimethylformamide and 2 ml of pyridine, and it is mixed with 0.95 g (3.6 mmol) of

disuccinimidyl carbonate. After two hours, 1.4 ml (10 mmol) of triethylamine and 322 mg (1.8 mmol) of glucamine are added. It is stirred overnight at room temperature, the batch is evaporated to the dry state, and the residue is absorptively precipitated with diethyl ether. The solid is suctioned off and chromatographed on RP-material for purification.

Yield: 0.67 g (74% of theory)

Analysis (relative to solventless substance):

Cld: C 52.58 H 6.82 N 5.57 S 6.38 O 28.65

Fnd: C 52.47 H 6.91 N 5.39 S 6.44

b) 4-[2-[4-Chloro-7-[5-N-(2,3,4,5,6-pentahydroxyhexyl)aminocarbonyl-3,3-dimethyl-1-(4-sulfonatobutyl)indolin-2-ylidene]-3,5-(propane-1,3-diyl)-1,3,5-heptatrien-1-yl]-3,3-dimethyl5-[N-(2,3,4,5,6-pentahydroxyhexyl)aminocarbonyl](3H)indolio]butanesulfonate, sodium salt

A solution of 180 mg (0.5 mmol) of N-[5-anilino-3-chloro-2,4-(propane-1,3-diyl)-2,4-pentadien-1-ylidene]anilinium-chloride, 503 mg (1 mmol) of 5-N-(2,3,4,5,6-pentahydroxyhexyl)aminocarbonyl-1-(4-sulfobutyl)-2,3,3-trimethyl(3H)indolenine (Example 45a), and 165 mg (2 mmol) of anhydrous sodium acetate in 10 ml of ethanol are refluxed for two hours under argon. Then, the ethanol is distilled off, and the residue is purified by chromatography.

Yield: 0.31 g (53% of theory)

Analysis (relative to solventless substance):

Cld: C 54.05 H 6.33 Cl 3.01 N 4.76 S 5.44 O 24.45 Na 1.95

Fnd: C 53.89 H 6.20 Cl 2.87 N 4.83 S 5.29 Na 1.72

c) 4-[2-[4-(4-Isothiocyanatothiophenyloxy)-7-[5-N-(2,3,4,5,6-pentahydroxyhexyl)aminocarbonyl-3,3-dimethyl-1-(4-sulfonatobutyl)indolin-2-ylidene]-3,5-(propane-1,3-diyl)-1,3,5-heptatrien-1-yl]-3,3-dimethyl-5-[N-(2,3,4,5,6-pentahydroxyhexyl)aminocarbonyl](3H)indolio]butanesulfonate, sodium salt

54 mg (0.4 mmol) of 4-aminothiophenol is dissolved in 10 ml of absolute N,N-dimethylformamide under argon atmosphere and mixed at room temperature with 165 mg (0.14 mmol) of 4-[2-[4-chloro-7-[5-N-(2,3,4,5,6-pentahydroxyhexyl)aminocarbonyl-3,3-dimethyl-1-(4-sulfonatobutyl)indolin-2-ylidene]-3,5-(propane-1,3-diyl)-1,3,5-heptatrien-1-yl]-3,3-dimethyl-5-[N-(2,3,4,5,6-pentahydroxyhexyl)aminocarbonyl](3H)indolio]butanesulfonate, sodium salt (Example 45b). After 10 minutes, the reaction mixture is quenched with dry ice, and 210 mg (1 mmol) of thiocarbonyldiimidazole is added. After 45 minutes, the dye is precipitated with diethyl ether, and the solid is isolated via centrifuging. For purification, it is can be chromatographed on RP-material.

Yield: 78 mg (43% of theory)

Analysis (relative to solventless substance):

Cld:	C 55.07	H 6.01	N 5.35	S 9.80	O 22.01	Na 1.76
Fnd:	C 54.89	H 6.20	N 5.24	S 9.58		Na 1.54

Analogously to the synthesis according to Examples 14-16 and 18-27a (solid-phase peptide synthesis), the VIP-analog HSWAVFTDNY TRLRKQMAVK KYLNSILN(SEQ ID NO: 73) is synthesized in the solid phase. The dye 4-[2-[4-(4-isothiocyanatothiophenyloxy)-7-[5-N-(2,3,4,5,6-pentahydroxyhexyl)-aminocarbonyl-3,3-dimethyl-1-(4-sulfonatobutyl)indolin-2-ylidene]-3,5-(propane-1,3-diyl)-1,3,5-heptatrien-1-yl]-3,3-dimethyl-5-[N-(2,3,4,5,6-pentahydroxyhexyl)aminocarbonyl](3H)indolio]butanesulfonate, sodium salt, is coupled to the peptide in the N-terminal position, and this conjugate is isolated and purified according to Examples 14-16 and 18-27c (protective group cleavage and detachment of the dye-peptide conjugates).

Analogously, other symmetrical hydrophilic dyes can be built up from the following components:

Hydrophilic indolenine derivatives with hydroxyalkyl substituents: (produced according to Example 44a)

a) 5-N-(2,3-Dihydroxypropyl)-N-methylaminocarbonyl-1-(4-sulfobutyl)-2,3,3-trimethyl(3H)indolenine

b) 5-N-(Hydroxyethyl)-aminocarbonyl-1-(4-sulfobutyl)-2,3,3-trimethyl(3H)indolenine

c) 5-N-(2,3-Dihydroxypropyl)-N-(hydroxyethyl)-aminocarbonyl-1-(4-sulfobutyl)-2,3,3-trimethyl(3H)indolenine

d) 5-N,N-(bis-Hydroxyethyl)-aminocarbonyl-1-(4-sulfobutyl)-2,3,3-trimethyl(3H)indolenine

e) 5-N-(2,3,4,5,6-Pentahydroxyhexyl)-aminocarbonyl-1-(4-sulfobutyl)-2,3,3-trimethyl(3H)indolenine

f) 5-N-(1,3,4-Trihydroxybut-2-yl)-N-methylaminocarbonyl-1-(4-sulfobutyl)-2,3,3-trimethyl(3H)-indolenine

Example 46

7-[5-N-(2,3-Dihydroxypropyl)aminocarbonyl-3,3-dimethyl-1-(4-sulfonatobutyl)indolin-2-ylidene]-1,3,5-heptatrien-1-yl]-3,3-dimethyl-5-carboxy(3H)indolio]butanesulfonate, sodium salt

A solution of 2.35 g (5.71 mmol) of 5-N-(2,3-dihydroxypropyl)aminocarbonyl-1-(4-sulfobutyl)-2,3,3-trimethyl(3H)indolenine (Example 44a) and 1.57 g (5.5 mmol) of glutaconaldehyde dianilide-hydrochloride in 25 ml of acetic acid anhydride are stirred for 30 minutes at 120°C. Then, 2.4 g (7.1 mmol) of 5-carboxy-1-(4-sulfobutyl)-2,3,3-trimethyl(3H)-indolenine, 1.71 g of sodium acetate, 22 ml of acetic acid anhydride and 8.6 ml of acetic acid are added. The reaction mixture is allowed to stir for one hour at 120°C, cooled to room temperature, and the product is precipitated with diethyl ether. The crude product is chromatographed on RP-material.

Yield: 1.9 g (40% of theory)

Analysis (relative to solventless substance):

Cld:	C 54.47	H 6.03	N 5.03	S 7.67	O 21.05	Na 2.75
Fnd:	C 54.26	H 6.12	N 5.00	S 7.49		Na 2.48

Analogously to the synthesis according to Examples 14-16 and 18-27a (solid-phase peptide synthesis), the VIP-analog HSDAVFTDNY TRLRKKMAVK KYLNSILN(SEQ ID NO: 101) is synthesized in the solid phase. The dye 7-[5-N-(2,3-dihydroxypropyl)aminocarbonyl-3,3-dimethyl-1-(4-sulfonatobutyl)indolin-2-ylidene]-1,3,5-heptatrien-1-yl]-3,3-dimethyl-5-carboxy(3H)indolio]butanesulfonate, sodium salt, is coupled to the peptide in the N-terminal position according to Examples 14-16 and 18-27b (dye coupling), and this conjugate is isolated and purified using HPLC according to Examples 14-16 and 18-27c (protective group cleavage and detachment of the dye-peptide conjugate).

Analogously, other unsymmetrical hydrophilic dyes can be built up from the following components:

Hydrophilic indolenine derivatives with hydroxyalkyl substituents: (produced according to Example 44a)

a) 5-N-(2,3-Dihydroxypropyl)-N-methylaminocarbonyl-1-(4-sulfobutyl)-2,3,3-trimethyl(3H)indolenine

b) 5-N-(Hydroxyethyl)-aminocarbonyl-1-(4-sulfobutyl)-2,3,3-trimethyl(3H)indolenine

c) 5-N-(2,3-Dihydroxypropyl)-N-(hydroxyethyl)-aminocarbonyl-1-(4-sulfobutyl)-2,3,3-trimethyl(3H)indolenine

d) 5-N,N-(bis-Hydroxyethyl)-aminocarbonyl-1-(4-sulfobutyl)-2,3,3-trimethyl(3H)indolenine

e) 5-N-(2,3,4,5,6-Pentahydroxyhexyl)-aminocarbonyl-1-(4-sulfobutyl)-2,3,3-trimethyl(3H)indolenine

f) 5-N-(1,3,4-Trihydroxybut-2-yl)-N-methylaminocarbonyl-1-(4-sulfobutyl)-2,3,3-trimethyl(3H)indolenine

Indolenine derivatives with carboxyl groups:

a) 5-Carboxy-1-(4-sulfobutyl)-2,3,3-trimethyl(3H)indolenine

b) 5-Carboxymethyl-1-(4-sulfobutyl)-2,3,3-trimethyl(3H)indolenine

c) 5-Carboxy-1-(3-sulfopropyl)-2,3,3-trimethyl(3H)indolenine

d) 5-Carboxymethyl-1-(3-sulfopropyl)-2,3,3-trimethyl(3H)indolenine

e) 5-Carboxy-1-(2-sulfoethyl)-2,3,3-trimethyl(3H)indolenine

f) 5-Carboxymethyl-1-(2-sulfoethyl)-2,3,3-trimethyl(3H)indolenine

Dianil derivatives for reaction with the above-mentioned indolenines with the formation of mono-, di- or tricarbocyanines:

a) Glutaconaldehyde dianilide-hydrochloride

b) Malonaldehyde-bis-phenylimine-hydrochloride

c) N,N-Diphenylformamidine

d) N-[5-Anilino-2,4-(propane-1,3-diyl)-2,4-pentadien-1-ylidene]anilinium-chloride

e) N-[5-Anilino-2,4-(ethane-1,2-diyl)-2,4-pentadien-1-ylidene]anilinium-chloride

f) N-[5-Anilino-3-chloro-2,4-(propane-1,3-diyl)-2,4-pentadien-1-ylidene]anilinium chloride

g) N-[5-Anilino-3-chloro-2,4-(ethane-1,2-diyl)-2,4-pentadien-1-ylidene]anilinium chloride

Example 47

7-[5-N-(1,3,4-Trihydroxybut-2-yl)-aminocarbonyl-3,3-dimethyl-1-(4-sulfonatobutyl)indolin-2-ylidene]-3,5-(2-carboxypropane-1,3-diyl)-1,3,5-heptatrien-1-yl]-3,3-dimethyl-5-N-(1,3,4-trihydroxybut-2-yl)-aminocarbonyl(3H)indolio]butanesulfonate, sodium salt, N-hydroxysuccinimide ester

a) 4-[2-[4-Chloro-7-[5-N-(1,3,4-trihydroxybut-2-yl)aminocarbonyl-3,3-dimethyl-1-(4-sulfonatobutyl)indolin-2-ylidine]-3,5-[2-(methoxycarbonyl)propane-1,3-diyl]-1,3,5-heptatrien-1-yl]-3,3-dimethyl-5-[N-(1,3,4-trihydroxybut-2-yl)aminocarbonyl](3H)indolio]butanesulfonate, sodium salt

5.5 ml (10 mmol) of phosphoroxy chloride and 6 ml of N,N-dimethylformamide are added to a solution of 0.8 g (5 mmol) of 4-(methoxycarbonyl)-cyclohexanone in 5 ml of dichloromethane at 0°C. Then, it is refluxed for one hour. The dichloromethane is distilled off, and 4 ml of aniline in 10 ml of methanol is added at a maximum of 5°C. The reaction mixture is poured onto ice, 5 ml of concentrated hydrochloric acid is added, and the intermediate product is allowed to crystallize out for five hours at 0°C. The crystals are suctioned off, and the latter are used without further purification in the next reaction. To this end, the crystals are dissolved in anhydrous ethanol, and 4.4 g (10 mmol) of 5-N-(1,3,4-trihydroxybut-2-yl)-N-methylaminocarbonyl-1-(4-sulfobutyl)-2,3,3-trimethyl(3H)indolenine (see Example 45) and 0.8 g of anhydrous sodium acetate are added. It is refluxed for

one hour, the solid is filtered off, and the filtrate is evaporated to the dry state. The residue is chromatographed for purification.

Yield: 3.25 g (58% of theory)

Analysis (relative to solventless substance):

Cld:	C 54.90	H 6.14	Cl 3.18	N 5.02	S 5.75	O 22.94	Na 2.06
Fnd:	C 54.76	H 6.03	Cl 2.99	N 4.91	S 5.60		Na 1.83

b) 7-[5-N-(1,3,4-Trihydroxybut-2-yl)aminocarbonyl-3,3-dimethyl-1-(4-sulfonatobutyl)indolin-2-ylidene]-3,5-(2-carboxypropane-1,3-diyl)-1,3,5-heptatrien-1-yl]-3,3-dimethyl-5-N-(1,3,4-trihydroxybut-2-yl)aminocarbonyl(3H)indolio]butanesulfonate, sodium salt

A mixture of 10 mg (0.4 mmol) of sodium hydride and 80 mg (1.3 mmol) of ethanethiol in 10 ml of anhydrous N,N-dimethylformamide are stirred under nitrogen for 30 minutes at room temperature. Then, it is mixed with 112 mg (0.1 mmol) of 4-[2-[4-chloro-7-[5-N-(2,3,4,5,6-pentahydroxyhexyl)aminocarbonyl-3,3-dimethyl-1-(4-sulfonatobutyl)indolin-2-ylidene]-3,5-[2-(methoxycarbonyl)propane-1,3-diyl]-1,3,5-heptatrien-1-yl]-3,3-dimethyl5-[N-(2,3,4,5,6-pentahydroxyhexyl)-aminocarbonyl](3H)indolio]butanesulfonate, sodium salt (Example 47a), in 3 ml of N,N-dimethylformamide. The reaction mixture is heated for two hours to 100°C and after cooling to room

temperature, it is quenched with carbon dioxide. It is evaporated to the dry state in a rotary evaporator, and the residue is extracted with hot ethanol. The extract is evaporated, and the crude product is chromatographed.

Yield: 75 mg (67% of theory)

Analysis (relative to solventless substance):

Cld:	C 56.27	H 6.33	N 5.25	S 6.01	O 23.99	Na 2.15
Fnd:	C 56.13	H 6.46	N 5.12	S 5.87		Na 1.88

c) 7-[5-N-(1,3,4-Trihydroxybut-2-yl)-aminocarbonyl-3,3-dimethyl-1-(4-sulfonatobutyl)indolin-2-ylidene]-3,5-(2-carboxypropane-1,3-diyl)-1,3,5-heptatrien-1-yl]-3,3-dimethyl-5-(N-(1,3,4-trihydroxybut-2-yl)-aminocarbonyl(3H)indolio]butanesulfonate-N-hydroxysuccinimide ester, sodium salt

For the production of the active ester, 14 mg (0.12 mmol) of N-hydroxysuccinimide and 64 mg (0.06 mmol) of the carboxylic acid in 2 ml of N,N-dimethylformamide are dissolved. After 15 minutes, 25 mg (0.12 mmol) of dicyclohexylcarbodiimide is added, and it is stirred overnight at room temperature. The active ester is purified using preparative HPLC.

Yield: 60 mg (86% of theory)

Analysis (relative to solventless substance):

Cld:	C 55.71	H 6.06	N 6.02	S 5.51	O 24.73	Na 1.97
Fnd:	C 55.59	H 6.21	N 5.93	S 5.37		Na 1.75

Analogously to the synthesis according to Examples 14-16 and 18-27a (solid-phase peptide synthesis), the VIP-analog HSDAVFTDNY TRLRKAMAVK KYLNSILN(SEQ ID NO: 98) is synthesized in the solid phase. The dye 7-[5-N-(1,3,4-trihydroxybut-2-yl)-aminocarbonyl-3,3-dimethyl-1-(4-sulfonatobutyl)indolin-2-ylidene]-3,5-(2-carboxypropane-1,3-diyl)-1,3,5-heptatrien-1-yl]-3,3-dimethyl-5-N-(1,3,4-trihydroxybut-2-yl)-aminocarbonyl(3H)indoliolbutanesulfonate, sodium salt, is coupled to the peptide in the N-terminal position according to Examples 14-16 and 18-27b, and this conjugate is isolated and purified according to Examples 14-16 and 18-27c (protective group cleavage and detachment of the dye-peptide conjugates).

Example 48

7-[5-N-(1,3,4-Trihydroxybut-2-yl)-aminocarbonyl-3,3-dimethyl-1-(4-sulfonatobutyl)indolin-2-ylidene]-3,5-(2-carboxypropane-1,3-diyl)-1,3,5-heptatrien-1-yl]-3,3-dimethyl-5-N-(1,3,4-trihydroxybut-2-yl)-aminocarbonyl(3H)indolio]butanesulfonate, sodium salt

a) 5-N-(11-Aminoundecyl)aminocarbonyl-1-(4-sulfobutyl)-2,3,3-trimethyl(3H)indolenine

340 mg (1 mmol) of 5-carboxy-1-(4-sulfobutyl)-2,3,3-trimethyl(3H)indolenine (Anal. Biochem. 217, 197, 1994) is introduced into 5 ml of absolute N,N-dimethylformamide and 1 ml of pyridine, and it is mixed with 0.5 g (2 mmol) of disuccinimidyl carbonate. After three hours, 0.805 g (4 mmol) of 11-aminoundecanoic acid is added. It is stirred overnight at room temperature, the batch is evaporated to the dry state, and the residue is absorptively precipitated with diethyl ether. The solid is suctioned off and chromatographed on RP-material for purification.

Yield: 0.37 g (71% of theory)

Analysis (relative to solventless substance):

Cld:	C 62.04	H 8.10	N 5.36	S 6.13	O 18.37
Fnd:	C 61.88	H 8.23	N 5.17	S 6.02	

b) 7-[5-N-(1,3,4-Trihydroxybut-2-yl)-aminocarbonyl-3,3-dimethyl-1-(4-sulfonatobutyl)indolin-2-ylidene]-3,5-(2-carboxypropane-1,3-diyl)-1,3,5-heptatrien-1-yl]-3,3-dimethyl-5-N-(1,3,4-trihydroxybut-2-yl)- aminocarbonyl-(3H)indolio]butanesulfonate, sodium salt

A solution of 0.35 g (0.67 mmol) of 5-N-(11-aminoundecyl)aminocarbonyl-1-(4-sulfobutyl)-2,3,3-trimethyl(3H)indolenine (Example 48a) and 0.18 g (0.645 mmol) of glutaconaldehyde dianil in 3 ml of acetic acid anhydride is

stirred for 20 minutes at 110°C. Then, 344 mg (0.83 mmol) of 5-N-(2,3-dihydroxypropyl)-aminocarbonyl-1-(4-sulfobutyl)-2,3,3-trimethyl(3H)indolenine (Example 44a), 0.2 g of sodium acetate, 3 ml of acetic acid anhydride and 1 ml of acetic acid are added. The reaction mixture is allowed to stir for two hours at 110°C, cooled to room temperature, and the product is precipitated with diethyl ether. The crude product is chromatographed on RP-material.

Yield: 0.41 g (60% of theory)

Analysis (relative to solventless substance):

Cld:	C 60.10	H 7.02	N 5.50	S 6.29	O 18.84	Na 2.26
Fnd:	C 59.96	H 7.14	N 5.33	S 6.15		Na 2.11

Analogously to the synthesis according to Examples 14-16 and 18-27a (solid-phase peptide synthesis), the VIP-analog HSDAVFTDNY TRLRKQMWVK KYLNSILN(SEQ ID NO: 123) is synthesized in the solid phase. The dye 7-[5-N-(1,3,4-trihydroxybut-2-yl)-aminocarbonyl-3,3-dimethyl-1-(4-sulfonatobutyl)indolin-2-ylidene]-3,5-(2-carboxypropane-1,3-diyl)-1,3,5-heptatrien-1-yl]-3,3-dimethyl-5-N-(1,3,4-trihydroxybut-2-yl)-aminocarbonyl(3H)indolio]butanesulfonate, sodium salt, is coupled to the peptide in the N-terminal position according to Examples 14-16 and 18-27b, and this conjugate is isolated and purified

according to Examples 14-16 and 18-27c (protective group cleavage and detachment of the dye-peptide conjugates).

Other dyes according to the invention can be produced according to Examples 44a and b, in which instead of 11-aminoundecanoic acid in Example 48a, the following amino acids are used, and the indolenine derivatives that are mentioned in Example 46 are used with hydroxyalkyl substituents a)-f):

i. Glycine

ii. Alanine

iii. ß-Alanine

iv. 4-Aminobutanoic acid

v. 6-Aminohexanoic acid

vi. $H_2N-(CH_2CH_2O)_3CH_2COOH$ (TH 53, 20, 6977)

vii. $H_2N-(CH_2CH_2O)_4CH_2COOH$ (JOC 63, 5, 1728, 1998)

viii. $H_2N-CH_2CH_2COO(CH_2CH_2O)_4-CO-CH_2CH_2COOH$ (Lett. Pept. Sci. 6, 135, 1999)

ix. HCl^{*} H_2N-PEG-COOH (MW 3400 g/mol; Shearwater Polymers Inc., USA

Example 49

4-[2-[4-(4-(N-(4-Aza-6-bromo-5-oxohexyl)aminocarbonyl-ethyl)phenyloxy)-7-[5-N-(dihydroxypropyl)aminocarbonyl-3,3-dimethyl-1-(4-sulfonatobutyl)indolin-2-ylidene]-3,5-(propane-1,3-diyl)-1,3,5-heptatrien-1-yl]-5-N-(dihydroxypropyl)aminocarbonyl-3,3-dimethyl(3H)indolio]butanesulfonate, sodium salt

a) [3-[N-(tert-Butoxycarbonyl)amino]propyl]-N'-(bromoacetyl)-amide

2.5 g (14.4 mmol) of [3-[N-(tert-butoxycarbonyl)amino]-propyl]amine is dissolved in 15 ml of dioxane, and after 4.4 ml of triethylamine is added at 0°C, it is mixed with 3.2 g (16 mmol) of bromoacetyl bromide. It is stirred overnight at room temperature, and then another 320 mg of bromoacetyl bromide is added. After two hours at room temperature, the precipitate is suctioned off, the solution is concentrated by evaporation, and the residue is taken up in ethyl acetate. It is washed with water, and the organic phase is dried on sodium sulfate.

Yield: 3.2 g (75% of theory)

Analysis (relative to solventless substance):

Cld:	C 40.69	H 6.49	Br 27.07	N 9.49	O 16.26
Fnd:	C 40.50	H 6.37	Br 26.89	N 9.58	

b) (3-[N-(Bromoacetyl)amino]propyl]amine, hydrochloride

3.1 g (10.5 mmol) of [3-[N-(tert-butoxycarbonyl)amino]propyl]-N'-(bromoacetyl)-amide (Example 49a) is stirred with 50 mmol of 1M hydrochloric acid in ethyl acetate for five hours at room temperature. The product is suctioned off, and the solid is rewashed with ethyl acetate.

Yield: 2.3 g (95% of theory)

Analysis (relative to solventless substance):

Cld:	C 25.94	H 5.22	Cl 15.31	Br 34.51	N 12.10	O 6.91
Fnd:	C 25.76	H 5.41	Cl 15.55	Br 34.34	N 11.97	

c) 4-[2-[4-(4-(N-(4-Aza-6-bromo-5-oxo-hexyl)aminocarbonyl-ethyl)phenyloxy)-7-[5-N-(dihydroxypropyl)amino-carbonyl-3,3-dimethyl-1-(4-sulfonatobutyl)indolin-2-ylidene]-3,5-(propane-1,3-diyl)-1,3,5-heptatrien-1-yl]-5-N-(dihydroxypropyl)aminocarbonyl-3,3-dimethyl(3H)indolio]butanesulfonate, sodium salt

121 mg (0.1 mmol) of 4-[2-[4-(4-(2-carboxyethyl)phenyloxy)-7-[5-N-(dihydroxypropyl)aminocarbonyl-3,3-dimethyl-1-(4-sulfonatobutyl)indolin-2-ylidene]-3,5-(propane-1,3-diyl)-1,3,5-heptatrien-1-yl]-5-N-(dihydroxypropyl)-aminocarbonyl-3,3-dimethyl(3H)indolio]butanesulfonate, sodium salt, N-hydroxysuccinimide ester (Example 44c) are dissolved in 0.5 ml of N,N-dimethylformamide, and mixed with 0.06 ml of triethylamine and 70 mg (0.3 mmol) of [3-[N-(bromoacetyl)amino]propyl]amine, hydrochloride (Example 49b).

It is stirred for four hours at 60°C, then cooled to room temperature, and the product is precipitated with diethyl ether. The solid is suctioned off and washed with ample diethyl ether.

Yield: 0.11 mg (80% of theory)

Analysis (relative to solventless substance):

Cld: C 56.17 H 6.18 Br 6.13 N 6.44 S 4.92 Na 1.76 O 18.40

Fnd: C 55.96 H 6.26 Br 6.01 N 6.27 S 4.81 Na 1.53

Analogously to the synthesis according to Examples 14-16 and 18-27a (solid-phase peptide synthesis), the VIP-analog HSDAVFTDNY TRLRKQCAVK KYLNSLLN(SEQ ID NO: 189) is synthesized in the solid phase. The dye bromide 4-[2-[4-(4-(N-(4-aza-6-bromo-5-oxo-hexyl)aminocarbonylethyl)phenyloxy)-7-[5-N-(dihydroxypropyl)aminocarbonyl-3,3-dimethyl-1-(4-sulfonatobutyl)indolin-2-ylidene]-3,5-(propane-1,3-diyl)-1,3,5-heptatrien-1-yl]-5-N-(dihydroxypropyl)amino-carbonyl-3,3-dimethyl(3H)indolio]butanesulfonate, sodium salt, is coupled to the peptide in the N-terminal position according to Example 17, and this conjugate is isolated and purified according to Examples 14-16 and 18-27c (protective group cleavage and detachment of the dye-peptide conjugates).